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CONTENTS

Bacterial polysaccharides: The nature and function of the exudate produced by Xanthomonas phaseoli — J. G. Leach, V. G. Lilly, H. A. Wilson, and M. R. Purvis, Jr. 113
Amino acid nutrition of Alternaria solani — Ralph W. Lewis 121
Altering disease resistance with ionizing radiation and growth substances — Paul E. Waggoner and A. E. Dimond 125
Fungi associated with the flowers, pods, and seeds of soybeans — R. A. Kilpatrick 131
Pathogenic variability within cleistothecia of Erysiphe graminis — H. R. Powers, Jr., and J. G. Moseman 136
Effects of insecticides and physical barriers on field spread of pepper veinbanding mosaic virus — John N. Simons 139
Three strains of cucumber mosaic virus affecting bell pepper in the Everglades area of south Florida — John N. Simons 145
Methods for estimating numbers of soil microorganisms lytic to fungi — H. Paul Carter and John L. Lockwood 151
Lysis of fungi by soil microorganisms and fungicides including antibiotics — H. Paul Carter and John L. Lockwood 154
Influence of the stubby-root nematode on growth of alfalfa — Ivan J. Thomason and S. A. Sher 159
Observations on the longevity without food of the burrowing nematode — Wray Birchfield 161
Relations between growth and respiratory metabolism is safflower infected by Puccinia carthami — J. M. Daly and R. M. Sayre 163
Rate and timing of fungicidal soil treatments — W. H. Cram and O. Vaartaja 169
Factors affecting symptom expression in Fusarium wilt of Dianthus — John R. Hood and Robert N. Stewart 173
Suppression by two substituted carbamates of symptoms induced by southern bean mosaic virus — F. M. Porter, B. C. Smale, W. H. Preston, Jr., and J. W. Mitchell 179
Damping-off of Ladino clover and Lespedeza as affected by soil moisture and temperature — J. H. Graham, V. G. Sprague, and R. R. Robinson 182
Phytopathological Note:
The culture of Phytophthora infestans — H. David Thurston 186
News

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BACTERIAL POLYSACCHARIDES: THE NATURE AND FUNCTION OF THE EXUDATE PRODUCED BY XANTHOMONAS PHASEOLI[1]

J. G. Leach, V. G. Lilly, H. A. Wilson, and M. R. Purvis, Jr.

SUMMARY

Xanthomonas phaseoli, the bean blight pathogen, produces a viscous matrix when grown on agar or in aerated liquid media containing glucose or certain other sugars. This substance is similar to the exudate formed in nature on affected bean plants. The physical and chemical characteristics of the exudate produced in culture lead to the conclusion that it is a heteropolysaccharide with a very high molecular weight (19,500,000) and with acidic properties. It behaves as a hydrophilic colloid, contains bound water, and is moderately hygroscopic. Appreciable numbers of cells of *X. phaseoli* survived for as long as 1325 days when stored in the exudate under a variety of conditions. The thermal death point of *X. phaseoli* is a few degrees higher in exudate than in nutrient broth alone. The ultraviolet absorption by crude exudate produced on agar indicates that in nature it may provide considerable protection against the injurious effects of light. The exudate caused a wilting of equal severity on excised seedlings of tomato, sunflower, and bean. Only concentrations with noticeable viscosity induced wilt. Because of the lack of specificity, the reversible nature of the wilting in early stages, and the correlation of wilting with low water uptake by excised seedlings, it is concluded that wilting is a result of mechanical blocking of water movement rather than of a specific chemical toxicity. The chemical and physical properties of the polysaccharide show clearly that it is quite different from that isolated from *X. phaseoli* by Feder and Ark and from that isolated from *Agrobacterium tumefaciens* by Hodgson, Riker, and Peterson.

Most plant pathogenic bacteria do not form spores, but many are remarkably resistant to desiccation and survive for relatively long periods under dry conditions. The bean-wilt pathogen (*Corynebacterium flaccumfaciens* (Hedges) Dows.) was shown by Hedges (8) to survive for nearly 5 years in "dried bacterial ooze." Burkholder (4) has reported the isolation of the same organism from under the seed coat of infected bean seeds after storage for 25 years at room temperature. This apparent resistance to desiccation is usually assumed to be due to the protective effect of the so-called "ooze" or exudate formed by the bacteria. Despite their recognized importance for the survival of plant pathogenic bacteria, the exudates themselves have received very little study from this standpoint. Although exudates are formed by numerous plant pathogenic bacteria, only that produced by *Erwinia amylovora* (Burr.) Winsl. et al has been studied extensively in relation to the survival of the pathogen.

For many years, it was assumed that *E. amylovora* was especially susceptible to desiccation, although Waters (22) in 1922 and Pierstoff (19) in 1931 had observed that the bacteria were able to survive for a considerable length of time in dried exudate. The most extensive studies of the role of the exudate in the survival of *E. amylovora* were made by Rosen (20) and Hildebrand (9). Rosen (20) in 1936 showed experimentally that bacteria could survive for more than a year in dried exudate held in a closed chamber over sulfuric acid at a relative humidity approaching 0 per cent. In 1938 (21) he reported on the survival in exudate stored at 4 different degrees of relative humidity (0, 9.5, 21, and 45 per cent), each at 5 different temperatures (16°, 25°, 30°, 35°, and 40°C). The bacteria survived for more than 300 days at all conditions, except those kept at 45 per cent relative humidity at 30°C or higher. The longest period of survival was at 9.5 per cent relative humidity at 30°, although the maximum survival at several other conditions was not determined because the test material for this study was exhausted after 400 days. In a later experiment, cells were found viable after 610 days storage in exudate kept at 0 per cent relative humidity at room temperature.

Rosen made no quantitative measurements of survival, but survival tests were made by placing "a droplet of the dried exudate" in 0.2 to 0.5 ml of sterile distilled water and using the suspension for inoculating young shoots of Bartlett pears. No counts were made of the viable cells in the original material nor of the number of bacteria surviving after storage.

Hildebrand (9) studied intensively the exudate of *E. amylovora* in 1939, but here also, no quantitative measurements of survival were made. Exudate produced on pear slices and dried on silk threads were stored in glass vials under different conditions. Virulent cultures were recovered from samples of dried exudate after periods ranging from 15 to 25 months, whereas the organism grown on nutrient agar never survived for as long as 1 month. Bacteria survived in moist exudate only 13 days. Hildebrand removed the bacteria from the exudate by centrifuging and filtering. When he added bacteria grown on agar slants to the sterile bacteria-free exudate and dried it on cotton threads, the bacteria survived for at least 3 months as compared to 13 days when suspended in

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distilled water and dried in the same way. Thus, the exudate imparted protection to cells grown on nutrient agar as well as to those formed in exudate on pear slices. Neither Rosen nor Hildebrand made extensive studies of the chemical or physical properties of the exudate.

The work reported here is part of a comprehensive study of bacterial exudates. The study was designed to yield information about the chemical and physical properties of the exudates as well as about their function. Particular attention has been given in this paper to their role in protecting plant pathogens against the injurious effects of desiccation and other adverse factors of the environment.

MATERIALS AND METHODS.—Preliminary studies of *E. amylovora* showed that it formed exudate readily on green pears or other suitable host tissue but formed very little in artificial culture on several media tried. At the same time, it was found that *Xanthomonas phaseoli* (E. F. Sm.) Dows. formed exudate not only on host tissue but also on glucose agar. Since exudate produced in quantity under controlled conditions on artificial media is better adapted to the study of its chemical and physical nature and to quantitative measurements of the survival of bacteria than are exudates formed on host tissues, *X. phaseoli* was chosen for further work.

The isolate of *X. phaseoli* used in this work was furnished by Dr. W. J. Zaumeyer, Plant Industry Station, Beltsville, Maryland. Because of the variety of experimental techniques used in this work, the detailed experimental methods are reported in connection with the individual experiments.

EXPERIMENTAL RESULTS.—Since a dependable supply of exudate was required, the first experiments were made to find the most suitable medium for its production.

Production of exudate.—1) *On agar medium.*—*X. phaseoli* grew well on nutrient agar with 5 g of glucose per liter and produced a copious exudate similar in appearance to that formed on beans in nature. The yield of crude moist exudate (including the bacterial cells) was 6.5 g on each 100 cm^2 of agar surface after 12 days' incubation at 25°C. Because of the uncertain chemical content of nutrient agar, it was replaced in subsequent experiments by the following synthetic basal medium: acid hydrolyzed casein equivalent to 2 g of casein; fumaric acid, 1.3 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; Na_2CO_3, 1.1 g; Fe^{+++} and Zn^{++}, 0.2 mg each; Mn^{++}, 0.1 mg; distilled water, 1000 ml, to which was added 25 g agar. To this basal medium a suitable source of carbon was added and the pH adjusted to 7.0 before sterilization.

Pyrex baking dishes (10 in. in diameter) with covers were used as culture vessels. After 500 ml of medium was added, the cover was replaced, and the dishes were enclosed in 2 paper sacks which were closed with paper clips. The prepared dishes were autoclaved for 30 minutes at 15 lb. pressure.

The inoculum was prepared by growing *X. phaseoli* on nutrient agar in 250-ml Erlenmeyer flasks. The cells were removed and suspended in sterile distilled water; sufficient cell suspension was used to cover evenly the surface of the medium in the baking dish. The culture dishes and paper sacks were then closed and the cultures incubated at 25°C.

Exudate produced under these conditions was butter-yellow in color and had the consistency of petrolatum. It had little or no odor and was essentially tasteless. Almost complete removal of exudate without any agar was effected by use of a rubber household spatula.

The yields of exudate on glucose agar were directly proportional to the glucose concentrations between 0.0 and 15 g/l for 12 days' incubation and between 0 and 20 g/l for 20 days' (Fig. 1). The efficiency with which glucose is converted into exudate decreased as the glucose concentration increased, especially for concentrations greater than 25 g/l. For routine production of exudate by this method, 25 g/l glucose was used. From 4 to 6 times as much exudate was produced in this medium as on nutrient agar with 0.5 per cent glucose. This is a suitable method for producing moderate amounts of exudate.

2) *In liquid medium.*—Exudate was produced effectively in liquid glucose-casein hydrolysate medium when aerated. Only a trace of exudate was formed in nonagitated media. When 250-ml Erlenmeyer flasks containing 25 ml of medium were seeded and placed on a Brunswick rotary shaker, prompt growth and exudate formation took place. The exudate could be precipitated from liquid medium by acetone, and the general properties of exudate produced in liquid medium appeared to be similar to those of exudate produced on agar medium. This method is more satisfactory than that described above for producing the exudate in quantity.

3) *With various carbon sources.*—The basal medium was modified in these experiments by omitting fumaric acid and sodium carbonate. Glucose and the other carbon sources were used at a rate that supplied 10 g

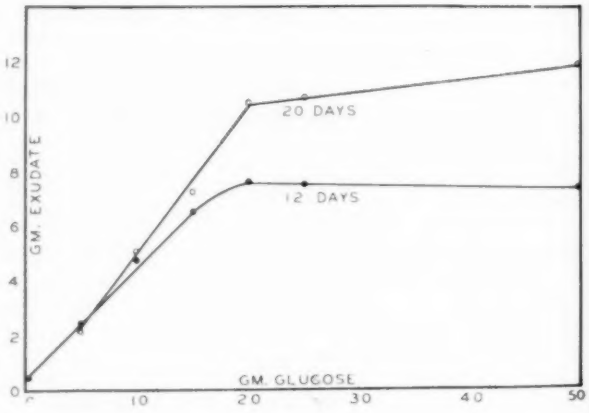

FIG. 1. Yield of exudate by *X. phaseoli* on casein-hydrolysate agar medium as a function of the glucose concentration after incubation for 12 and 20 days at 25°C.

of carbon per liter. Aliquots of the basal medium (less glucose) were added to weighed amounts of the carbon sources; 25-ml aliquots of these media were dispensed into 250-ml Erlenmeyer flasks. The flasks were capped with 50-ml Pyrex beakers rather than cotton plugs. Media were sterilized by autoclaving at 15 lb. pressure for 15 minutes. Each flask was seeded with 1.25 ml of a heavy cell suspension of *X. phaseoli*. The flasks were placed on a Brunswick rotary shaker and incubated at 25°C for 7 days.

Quantitative data on exudate production on the various carbon sources were obtained by pooling 3 flasks of a given medium and precipitating the exudate with excess acetone. The exudate was then transferred to aluminum weighing cups. The acetone was removed by evacuation in a vacuum desiccator, and the exudate was dried over night at 55°C. The yield was determined by weighing the dry exudate (Table 1). The entire experiment was repeated with concordant results.

The carbon source used in the medium is an important factor in the production of exudate. Glucose and mannose among the monosaccharides, and sucrose among the oligosaccharides, were the best carbon sources for the production of exudate under these conditions. Sorbitol and mannitol, which are sugar alcohols related to glucose and mannose, were poor carbon sources. On structural grounds, it would be expected that fructose would be equally as good as glucose and mannose but this was not true in this experiment. The failure of *X. phaseoli* to grow and produce exudate on fructose proved in later experiments to be due to the autoclaving of this sugar with the basal medium. When fructose was sterilized by filtration or autoclaved separately before being added to the basal medium, prompt growth of *X. phaseoli* and exudate formation followed. The toxicity of autoclaved media containing fructose has been noted for *Thielaviopsis basicola* (Berk. & Br.) Ferr. (3). *X. phaseoli* neither grew nor produced exudate on the pentose sugars or on L-sorbose. L-Sorbose is poorly utilized by many fungi (2, 14, 17).

TABLE 1.—*Milligrams of exudate produced in 25-ml aliquots of casein-hydrolysate medium containing different sources of carbon and incubated at 25°C on a rotary shaker for 7 days*

Carbon source	Experiment 1	Experiment 2
Cellobiose	52	47
D-Fructose [a]	0.0	0.0
D-Galactose	89	97
D-Glucose	128	141
Lactose	67	65
Maltose	72	80
Melibiose	61	49
Mannitol	13	7
D-Mannose	118	115
Raffinose	38	29
Sorbitol	17	18
Sucrose	173	161

[a] Fructose here autoclaved with the basal medium.

Green bean pods contain 3 sugars that are excellent carbon sources for exudate production. The presence of glucose, fructose, and sucrose in green bean pods was demonstrated by means of paper-strip chromatography.

Biological properties of the exudate.—1) *Survival in dried exudate.*—Since quantitative measurements of survival in dried exudate were planned, it was necessary to determine the number of viable bacteria in the original moist exudate. Counts were made by the standard procedures for plate counts (1); 0.85 per cent saline solution was used for dilutions, since it gave somewhat higher counts than were obtained when dilutions were made in distilled water. The plates were incubated at 25°C for 5 days, and the colonies were counted on a Quebec colony counter.

Preliminary counts made from exudate grown on nutrient agar containing 5 g of glucose per liter gave such erratic results that a series of counts were made to determine the nature of the growth curve on this medium. These counts showed that the peak concentration of viable bacteria in the exudate (197,000,000 per mg) was reached by the second day and that this concentration was maintained with only a slight drop until the seventh day, when the phase of accelerated death apparently began and the concentration of viable cells decreased rapidly. For this reason, all samples of exudate used for testing survival were taken before the seventh day and usually on the fourth day.

When glucose casein-hydrolysate agar with 25 g glucose per liter was adopted as a standard solid medium, a similar series of counts was made from exudate grown on this medium. Here again, the peak concentration was reached by the second day, but the number of viable bacteria (61,000,000 per mg) was only about one third of that on the nutrient agar with 5 g of glucose per liter. Also, the growth curve was quite different. The phase of accelerated death was much more gradual, there being a very slow decrease in concentration until there were still 10,000,000 viable cells per mg on the 15th day.

The lower concentration of viable bacteria in the exudate formed on the high-glucose agar was attributed to the greater amount of exudate being produced by the same number of bacteria. Likewise, the slower rate of death of cells after the peak concentration may have been due to the greater amount of exudate that surrounded each cell, giving the cells greater protection against injurious metabolic products. This explanation was supported by microscopic examination of the exudates produced on the 2 media; it was obvious that the number of cells was less, in proportion to the amount of exudate, on the agar with 25 g of glucose per liter than in that containing less glucose.

After considerable "trial and error" experimentation, the following procedures were adopted in making quantitative measurements of survival of *X. phaseoli* in its exudate. Cover glasses (⅞ × ⅞ in.) were

TABLE 2.—*Counts in millions of viable bacteria (X. phaseoli) per mg of moist exudate, made from drops of exudate produced on nutrient agar plus 0.5 per cent glucose after storage for given periods of time at different temperatures and relative humidities*

Days storage	Counts in millions (with percentage survival in parentheses) at indicated relative humidity		
	0% R.H.	20% R.H.	51% R.H.
	Temperature −25°C		
0	110.5[a]	110.5	110.5
11	73.0 (66)	66.0 (57)	95.5 (86)
100	47.4 (43)	75.0 (68)	88.5 (80)
312	16.0 (15)	37.0 (33)	57.7 (52)
589	15.9 (14)	16.2 (15)	33.7 (30)
1052	5.5 (5)	10.0 (9)	30.7 (28)
1325	3.3 (3)	4.8 (4)	6.3 (6)
	5°		
0	110.5[a]	110.5	110.5
18	29.0 (26)	71.8 (65)	65.7 (59)
102	5.5 (5)	34.0 (31)	2.6 (2)
313	0.8 (1)	10.5 (10)	0.0 (0)
560	1.8 (2)	7.3 (7)	0.0 (0)
1052	0.08 (0.1)	4.4 (4)	0.0 (0)
1325	0.20 (0.2)	0.3 (0.3)	0.0 (0)
	20°		
0	110.5[a]	110.5	110.5
14	2.0 (2)	11.4 (10)	2.3 (2)
101	0.2 (0.2)	0.1 (0.1)	0.9 (0.8)
312	0.2 (0.02)	1.0 (1.0)	0.04 (0.04)
560	0.002 (0.002)	0.4 (0.4)	0.0 (0)
1052	0.0 (0)	0.7 (0.07)	0.0 (0)
1325	0.0 (0)	0.02 (0.01)	0.0 (0)

[a] = Initial count.

cleaned and numbered with India ink and placed on dry filter paper in Petri dishes, 5 cover glasses to a dish. They were then sterilized by dry heat.

A cover glass was removed from the Petri dish and weighed on a chainomatic balance. A small mass of exudate (10–15 mg) was picked up with a sterile loop from a 4-day-old culture on glucose agar in a 250-ml flask and placed on the center of the cover glass. The scales were balanced promptly and the combined weight of the cover glass and exudate recorded. The cover glass was then replaced in the Petri dish. Petri dishes, each containing 5 cover glasses with weighed drops of exudate, were used in determining survival at different combinations of relative humidity and temperature. Bacterial counts were made on freshly weighed drops and on the dried drops after various treatments and periods of storage.

Desiccators providing each of 3 different relative humidities as indicated were prepared by filling the bottom chambers of each with one of the following materials: Drierite[2] (0 per cent R.H.), a saturated solution of potassium acetate (20 per cent R.H.), and a saturated solution of calcium nitrate (51 per cent R.H.).

Bacterial counts were made on nutrient agar after incubation at 25°C. In the making of dilutions for counting, a cover glass with the dried exudate was placed in 100 ml of sterile saline solution in a 250-ml flask and shaken on a Precision Equipoise shaker until the exudate was washed from the cover glass. All plates were poured within 20 minutes after dilution.

The first survival tests were made with exudate that had been produced on nutrient agar containing 0.5 per cent glucose. After the drops of exudate and cells were weighed, they were placed immediately in desiccators and stored at −25°, 5°, and 20°C for 1325 days. Representative data from these tests are given in Table 2. The second set of survival tests was made on exudate and cells grown on casein-hydrolysate agar containing 2.5 per cent glucose. Drops of this exudate were dried under vacuum over Drierite before being stored at different temperatures and relative humidities for 1143 days (Table 3).

In the first experiment, the numbers of cells that survived was greatest at −25°C. At this temperature, more cells survived at 51 per cent relative humidity than at either 20 or zero per cent relative humidity. At 5°, survival was better at 20 per cent relative humidity than at either zero or 51 per cent relative humidity. The cells stored at 20° died more rapidly than those stored at lower temperatures. At this temperature the best survival was at 20 per cent relative humidity.

In the second series, in which only 2 temperatures were used (−25° and 5°C), survival was better under all conditions for 1143 days than in the first test.

Since the exudates and cells used in these 2 longevity experiments were produced on different media, it is difficult to compare the results. Whether the better survival at 5° in the second experiment is to be attributed to the smaller number of cells per mg of exudate, to the effect of quick drying, or to other factors cannot be determined from the available evi-

TABLE 3.—*Counts in millions of viable cells of X. phaseoli per mg of moist exudate produced on casein hydrolysate agar plus 2.5 per cent glucose when drops of exudate and cells were quick-dried at room temperature before being stored at different temperatures and relative humidities*

Days storage	Counts in millions (with percentage survival in parentheses) at indicated relative humidity		
	0% R.H.	20% R.H.	51% R.H.
	Temperature −25°C		
0	15.8[a]	15.8	15.8
28	6.7 (42.4)	7.4 (46.8)	5.4 (34.2)
68	6.6 (41.8)	7.7 (48.7)	2.1 (13.3)
133	4.5 (28.5)	8.3 (52.5)	1.7 (10.8)
420	4.8 (30.4)	5.2 (32.9)	3.7 (23.4)
1143	2.1 (13.3)	1.4 (8.9)	1.3 (7.7)
	5°		
0	15.8[a]	15.8	15.8
29	6.2 (39.2)	8.5 (53.8)	4.9 (31.0)
69	4.9 (31.0)	7.2 (45.6)	3.3 (20.9)
133	2.6 (16.4)	3.0 (19.0)	4.1 (25.9)
402	5.2 (32.9)	5.9 (37.3)	0.53 (3.4)
883	2.3 (15.2)	0.34 (2.2)	0.05 (0.3)
1143	1.5 (9.5)	0.17 (1.1)	0.04 (0.2)

[a] = Initial count.

[2] Anhydrous calcium sulfate.

dence.

2) *Toxicity of exudate to excised seedlings.*—Recent investigations (5, 12) have shown that certain polysaccharides produced by plant pathogenic bacteria and fungi are phytotoxic and possess wilt-inducing properties. Feder and Ark (5) isolated toxic polysaccharides from *Agrobacter tumefaciens* (Smith & Town.) Conn, *X. phaseoli* (E. F. Sm.) Dows., and *Erwinia carotovora* (Jones) Holland but showed that they were not specific in their parasite and host-plant relationships. These polysaccharides were derived from within the cells rather than from exudates and were considered to be constituents of endotoxins. Nevertheless, it seemed worthwhile to determine whether the exudate produced by *X. phaseoli* possessed wilt-inducing properties. Purified acetone-precipitated exudate previously dried over Drierite and ground in a Wiley mill was diluted to form a 0.05 per cent solution, which was opalescent and obviously viscous. This solution was further diluted to give solutions of 0.005 and 0.0005 per cent. Greater concentrations were not used because of the viscosity. Excised tomato, sunflower, and bean seedlings were placed in triplicate 250-ml flasks of each of the solutions. Definite wilting occurred after 25 hours only at the 0.05 per cent concentration, indicating a relatively low degree of toxicity. There also was no significant difference in the response of the 3 kinds of seedlings, indicating a lack of specificity. Also, the plants regained their turgor when the exudate solution was replaced with distilled water in the early stages of wilt. The previously described wilt-producing polysaccharides are of relatively low molecular weight; however, as pointed out by Hodgson, Peterson, and Riker, (10) there is an increase in toxicity with increased molecular weight up to 50,000. On the assumption that hydrolysis of the exudate would decrease the size of the molecules as the viscosity decreased, a series of similar tests was included in which exudate that had been autoclaved for periods of 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, and 8 hours was used. Here also, wilting occurred only in the 0.05 per cent dilutions, indicating that this amount of hydrolysis did not influence the wilt-inducing properties of the exudate.

As will be pointed out later, the polysaccharides dealt with here have extremely large molecules (with a molecular weight of over 19,000,000), hence it is evident that the correlation between toxicity and size of the molecule does not occur in this case. The lack of specificity, the obvious viscosity of the exudate solutions, and the reversible nature of the early stages of wilting all point to the conclusion that the wilt-producing property of the exudate is due to mechanical occlusion of the normal pathways of water uptake and that beyond a certain point, the size of the molecule does not appreciably influence the water uptake.

In one experiment, the water uptake by the excised seedlings was measured over a 10-day period. The data show that the water uptake decreased with the increased concentration of the exudate solution (Table 4). At the higher concentration, water uptake was insufficient to maintain normal turgor.

TABLE 4. *Average water uptake by excised seedlings of tomato and sunflower with stems immersed for 10 days in given dilutions of exudate (dry basis)*

Exudate dilution	Tomato	Sunflower
per cent	ml	ml
0.05[a]	15	8
0.005	21	20
0.0005	28	25
0.00005	36	37
Water control	60	60

[a] Crude exudate dilutions calculated on dry-weight basis.

3) *Effect of exudate on thermal death point of X. phaseoli.*—The thermal death point was determined for *X. phaseoli* in exudate and in nutrient broth without glucose.

The following method was used: Five-day-old exudate produced on the surface of glucose-casein-hydrolysate agar was thoroughly mixed under aseptic conditions to obtain an even distribution of the included cells. The exudate was then placed in a sterile 100-ml hypodermic syringe and a 20-ml quantity forced into the bottom of each of several 20 mm × 150-mm sterile aluminum-capped test tubes. Water baths, set at the desired temperatures and with the water continuously agitated with motor stirrers, were used to maintain the desired temperatures during the determinations. Tubes containing 5-day-old exudate and a 48-hour culture in nutrient broth were placed in the water baths. As soon as the tube contents had reached the temperature of the surrounding water, zero time was recorded. Beginning at zero time and at 5-minute intervals for 1 hour, material from each tube was streaked upon the surface of solidified nutrient agar. The streaked plates were incubated for 5 days at 28°C and the amount of growth recorded. The results of 3 separate trials showed that the thermal death point in exudate was approximately 53°C as compared to 49–51°C in nutrient broth.

PHYSICAL AND CHEMICAL PROPERTIES OF THE EXUDATE.—Crude exudate produced on glucose-casein-hydrolysate agar medium formed an opalescent solution when diluted and agitated with water. When such a solution was treated with ethanol or acetone, a fibrous precipitate formed. When precipitated exudate was redissolved in water, the addition of ethanol induced the formation of a transparent gel. If a trace of sodium chloride, or other salt, was added at this stage, a fibrous precipitate was formed. Acetone was more suitable than ethanol for reprecipitating exudate from solution. Exudate produced in liquid culture had similar properties.

Exudate from agar surface or liquid medium was precipitated by the addition of an excess of acetone. The precipitated exudate was collected on a stirring rod. Attempts to dry exudate in this state resulted in a hard, dark-colored mass. Repeated washing of precipitated exudate with fresh acetone followed by re-

moval of the excess acetone by pressing the precipitate and then subjecting it to vacuum evacuation in a desiccator yielded fluffy fibrous material that was light tan to white in color. The exudate was finally dried at 55°C.

Moisture content.—The moisture content of crude exudate produced on agar media was determined by drying weighed samples at 100°C to constant weight. Crude exudate contained 96.5 per cent water according to this method. Similar samples were dried in a vacuum desiccator over Drierite until constant weight was attained. Crude exudate lost 93.0 per cent water when dried in this manner. The difference in water content as determined by these 2 methods is a measure of the bound water content of crude exudate; this amounts to 3.5 per cent.

Ash content.—Crude exudate produced on glucose-casein-hydrolysate agar medium contained between 10 and 11 per cent ash (dry basis). Treatment of a solution of exudate with Amberlite MB-3 (a mixed bed ion exchange resin, Rohm and Haas Company), reduced the ash content below 1 per cent. When solutions of either crude or precipitated exudate were treated with Amberlite MB-3[3] or with Dowex 50[4], the solution became distinctly acid: pH values as low as 2 were frequently attained. These results, in conjunction with other evidence to be presented later, suggest that *X. phaseoli* exudate is a high-molecular-weight substance having acidic properties.

Molecular weight.—A sample of exudate was diluted and passed twice through a Sharples supercentrifuge. This treatment removed essentially all of the bacterial cells. The centrifuged solution was evaporated to dryness. Through the courtesy of Dr. John B. Conn of Merck and Company, Inc., a weight-average molecular weight determination was made. The 2 following paragraphs are from Dr. Conn's report.

"We have succeeded in obtaining for you a weight-average molecular weight on the *Xanthomonas phaseoli* exudate which you supplied, but were unable to do anything with the material in the ultracentrifuge or electrophoresis apparatus. In both cases the tendency of the material to gel prevented formation of real boundaries. However, electrophoresis did demonstrate that the material has a pronounced anionic mobility in 0.1 ionic-strength-phosphate buffer at 0°.

"Molecular weight measurement was carried out by light-scattering in an American Instrument Company light-scattering microphotometer, using light of wave-length 4358 Å. Sample #1, which dispersed the more rapidly in water, was employed at a concentration C of 2.22×10^{-3} g/ml., for which the dispersion reading T was 0.212. Then m = T/HC, where

$$\frac{H\ 32^3\ No^2}{3N\ 4} \quad \frac{N\text{-}No^2}{C} = 1.552 \times 10^{-4}\ No^2 \quad \frac{N\text{-}No^2}{C}$$

[3] A mixture of synthetic ion-exchange resins.
[4] A synthetic cation exchange resin.

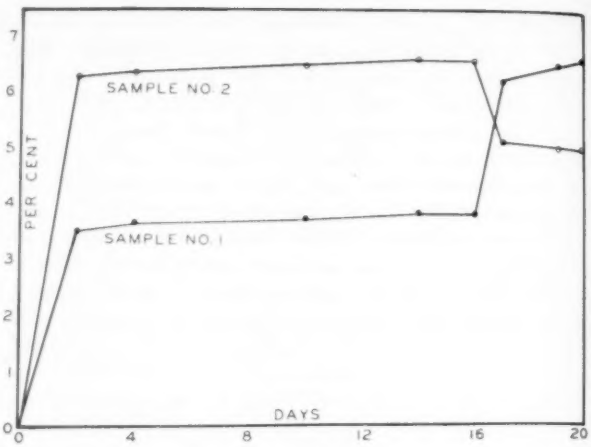

FIG. 2. Sorption and desorption of water vapor by ethanol-precipitated exudate. Sample No. 1 was kept at 15 per cent relative humidity for 16 days and then transferred to 20 per cent relative humidity. Sample No. 2 was kept at 20 per cent relative humidity for 16 days and then transferred to 15 per cent relative humidity.

The value for the refractive index difference between water and the solution (N-No) was obtained by means of a calibrated Zeiss interferometer, and found to be 2.97×10^{-4}. From these data, the molecular weight of the exudate was computed to be 19,500,000, a value not unexpected in view of the high viscosity in solution."

Hygroscopic properties.—A comparison was made of the ability of crude and ethanol-precipitated exudate to take up moisture from atmospheres of known relative humidity. Gram samples of dry exudates were weighed into glass-stoppered weighing bottles. The samples were placed in desiccators that contained saturated solutions of appropriate salts; an excess of the solid phase was maintained during the determinations. The desiccators were kept at 25°C and the samples were weighed frequently. These experiments were terminated at the end of 16 days. Equilibrium was attained at relative humidities of 15 and 20 per cent but not at higher relative humidities by the end of 16 days. Crude exudate took up more moisture at the higher relative humidities than did ethanol-precipitated exudate (Fig. 2). The reverse was true at lower relative humidities.

The reversible nature of moisture sorption and desorption was demonstrated in the following ways. The samples of alcohol-precipitated exudate that had reached equilibrium at relative humidities of 15 and 20 per cent were interchanged on the sixteenth day and the loss and gain in weight determined daily for 4 days. The sorption of moisture by the sample originally at equilibrium at 15 per cent relative humidity had apparently attained equilibrium by the end of 4 days when transferred to 20 per cent relative humidity (Fig. 2). The behavior of the samples originally at equilibrium at 20 per cent relative humidity on being transferred to 15 per cent relative humidity

TABLE 5.—*Relative viscosities of 1:799 (w/v) exudate solutions produced under 4 given conditions*

Sample	Relative viscosity
1. Crude exudate produced on glucose-casein-hydrolysate agar, 4 days old	2.44
2. Same as No. 1 except exudate was 32 days old at time of harvest	4.47
3. Acetone-precipitated exudate from liquid glucose-casein-hydrolysate medium 13 days old	6.38
4. Acetone-precipitated exudate from vigorously aerated medium containing glucose and enzymatic casein hydrolysate, 5 days old	8.24

suggests that the equilibrium of the moisture content depends upon the previous history of the sample. This is in accord with the results of Northcote (16), who found a difference in the amount of water sorbed or desorbed by Leuconostoc dextran and other polysaccharides at certain relative humidities. More water was retained in the process of desorption than gained in sorption.

Viscosity.—One of the most striking properties of solutions of the exudate from *X. phaseoli* is their viscosity. A 1 per cent (dry basis) solution is too viscous to pour easily.

Simple Ostwald-type viscosimeters were used to determine the relative viscosity of crude and acetone-precipitated exudate. Measurements were made at 30.0°C; the relative viscosities were calculated from flow times. Exudate solutions of 1:799 (dry-weight basis) were used in these experiments. Some of the results obtained in these experiments are reported in Table 5.

The relative viscosity of exudate from *X. phaseoli* is modified by the conditions under which it is produced. Presumably, these differences are due to variation in molecular weight. According to Nadel et al (15), the molecular weight and viscosity of the polysaccharide from *Leuconostoc mesenteroides* (Cienk.) v. Tiegh. varies depending upon the conditions under which it is produced.

Autoclaving at 1:799 (w/v) solution of acetone-precipitated exudate from *X. phaseoli* for 4 hours at 15 lb. pressure (121°C) reduced the relative viscosity from 8.24 to 1.67. An aliquot of the same solution was heated 4 hours at 92°C; the relative viscosity after this treatment was 4.29. In other experiments, it was noted that the acidity of autoclaved exudate solutions increased with increased times of autoclaving. The effect of autoclaving on the viscosity of 1:799 (w/v) solutions of another sample of acetone-precipitated exudate is shown in Figure 3.

Ultraviolet absorption.—The lethal effect of ultraviolet radiation on bacterial cells is well known. It seemed of interest to determine the absorptive power of the exudate from *X. phaseoli* for ultraviolet radiation of different wave lengths. Samples of crude exudate produced on glucose-casein-hydrolysate agar medium and acetone-precipitated exudate produced in liquid glucose-casein-hydrolysate medium were used. Suitable dilutions in distilled water for ultraviolet transmittance studies were found to be 1:10,000 (w/v). The determinations were made on a Beckman model DU spectrophotometer with quartz optics and cells, provided through the courtesy of Dr. D. T. Watts, Professor of Pharmacology, West Virginia University. Absorption was calculated by subtracting transmittance values from 100. Transmittance values were determined for 2-mμ intervals in the wave-length range from 220 to 310 mμ. Crude exudate was more efficient than acetone-precipitated exudate in absorbing ultraviolet radiation (Fig. 4). These data suggest that natural exudate produced in the field protects *X. phaseoli* from ultraviolet radiation in nature.

Chemical composition.—When acetone-precipitated exudate was fused with metallic sodium, the Lassaigne test for nitrogen was faint or doubtful. This suggests that the exudate contains no more than a trace of

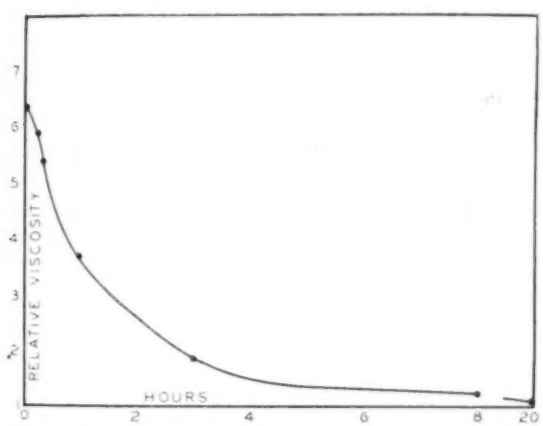

FIG. 3. The effect of time of autoclaving (121°C) on the relative viscosity of a 1:799 (w/v) solution of acetone-precipitated exudate of *X. phaseoli*.

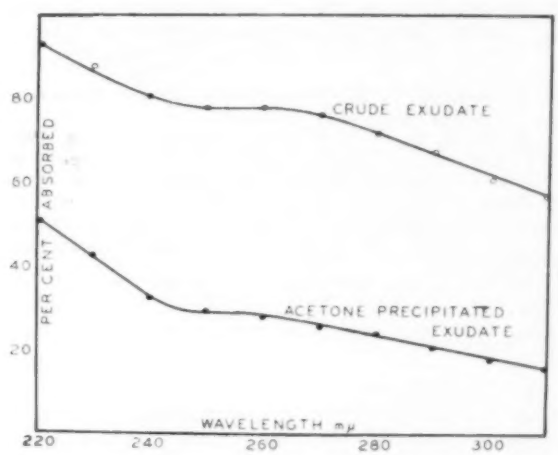

FIG. 4. Ultraviolet absorption by 1:10,000 (w/v) solutions of crude and acetone-precipitated exudate of *X. phaseoli* between 220 and 310 mμ.

nitrogen. When a solution of acetone-precipitated exudate was heated with Benedict's reagent, the test for reducing sugars was negative; however, if a solution of acetone-precipitated exudate was heated with acid, reducing substances were liberated as shown with Benedict's reagent.

A sample of acetone-precipitated exudate was hydrolyzed with 1 N sulfuric acid (6,7). After 3 hours autoclaving (121°C), the hydrolysate was treated with solid barium carbonate to pH 5. The filtrate and washings were concentrated and tested for sugars by descending filter paper chromatography according to the methods of Partridge (18). Two substances were found in the hydrolysate; these migrated at the same rate as glucose and mannose and gave the same colors as these sugars when aniline hydrochloride (13) and other sugar reagents were used. In addition, other reducing substances, presumably sugars and uronic acids, were present in the hydrolysate.

On the basis of the physical and chemical studies presented above, it is concluded that exudate from *X. phaseoli* is a high-molecular-weight, hydrophilic, colloidal substance. It appears to be an acidic heteropolysaccharide; although it is possible that the exudate is a mixture of homopolysaccharides. It is obvious that the polysaccharide described here is not the same as the endopolysaccharides isolated by Feder and Ark (5) from 3 plant pathogens including *X. phaseoli*. Its physical and chemical properties indicate also that it is different from that isolated from *Agrobacterium tumefaciens* by Hodgson, Riker, and Peterson (11, 12). Further data on the chemical properties and production of the exudate will be published in a later paper.

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AMINO ACID NUTRITION OF ALTERNARIA SOLANI[1]

Ralph W. Lewis

SUMMARY

Cystine and β-alanine are fairly constant inhibitors of growth of *Alternaria solani* Kühn. In the absence of vitamins, these inhibitions become stronger; in the presence of other amino acids, they usually are relieved. When high concentrations of proline are present, β-alanine increases growth. Other single amino acids inhibit less strongly, have no effect, or enhance growth. Combinations of 2 amino acids that singly enhance growth may further increase growth when present together (aspartic acid with arginine or with proline) or may give less growth than does either one of the amino acids (arginine with sodium glutamate). Two amino acids that inhibit growth, when used alone, will usually not inhibit in combination; occasionally, however, a pair inhibits growth more strongly than each alone. An example of the former is β-alanine with α-amino *n*-butyric acid, and an example of the latter is α-amino *n*-butyric acid with hydroxyproline. The fact that the great majority of amino acids, either singly or in combination, do not inhibit growth seems to be evidence against the balance hypothesis of parasitism. Since the media used in these experiments did not begin to approach in complexity the environment supplied by the host, the validity of the hypothesis remains uncertain.

Nutrition studies of microorganisms grown on synthetic media have established the fact that many combinations of foods that one would expect to support growth of an organism fail to do so.[2] Failure to grow may be due to the absence of an essential nutrient or nutrients, or may be due to the presence of a substance or substances that inhibit growth. These latter substances are, in the broad biological sense, considered to be foods, and are the normal metabolites of many organisms. The research reported here was carried out largely with the intent of getting nutrition facts about a common plant parasite to learn if such facts might indirectly shed some light on the nature of the host-parasite relationship.

MATERIALS AND METHODS.—The organism (*Alternaria solani* Kühn) and the methods used are the same as described in a previous paper on vitamin nutrition (3). In addition to glucose and inorganic foods, the basal medium contained D-biotin, 0.004 mg/l; choline chloride, 2.0 mg/l; and thiamine chloride, 1.0 mg/l. The amino acids were commercial grades supplied by General Biochemicals, Inc., and Nutritional Biochemicals Corp. As in the vitamin nutrition experiments, all cultures were in duplicate.. In all but a very few cases the differences between duplicates was less than 4 mg.

In stock-culture tubes of potato-dextrose agar, the fungus was sometimes variable. This was evident as a gradual loss of the normal greenish-grayish-black color of the mycelium or as lighter colored sectors. This variability was combatted by always transferring stock cultures from darker portions of the parent culture. Occasionally it was necessary to start cultures afresh by transferring from a culture kept under oil in the refrigerator.

Some variability in the results presented here may be ascribed to the fact that the experiments were conducted during all seasons of 3 years, 1951–1953. The variations in the temperature of the "constant-temperature" room were different in summer than in winter, even though the controls were carefully checked to keep the room at 27°C. Despite variations in the fungus and despite the fact that experiments were conducted over a long period of time, certain results have been exceedingly constant, and it is largely these that are reported.

Amino acids were added to basal medium (20 ml in a 125-ml Erlenmeyer flask) in 2 ml or less of water. The medium was then autoclaved 10 minutes at 15 lb. pressure. Unless otherwise stated, the cultures were grown for 72 hours at 27°C on a shaker as described previously (3). Whenever cystine or tyrosine was used, the amount for each flask was weighed separately and added directly to the flask.

EXPERIMENTS AND RESULTS.—*Effects of single amino acids.*—Alanine, arginine, aspartic acid, glutamic acid, glycine, and proline increased growth at the highest amounts used (Table 1). β-Alanine and cystine inhibited very strongly at the 5-mg and 15-mg levels. Except for histidine, monosodium glutamate, ornithine, serine, and threonine, the other amino acids inhibited more or less strongly at all 3 levels used. At 20 mg/20 ml, a higher level than used in these experiments, monosodium glutamate strongly increased growth (Table 2).

Effect of combining 2 growth-enhancing amino acids.—A series of experiments was designed to test the additive effects of arginine with sodium glutamate, of asparagine with proline, and of asparagine with arginine. Arginine with sodium glutamate had no additive effect (Table 2).

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TABLE 1.—*Growth of Alternaria solani on basal medium plus single amino acids*

Amino acid added	Increase in dry weight in 72 hours on media containing indicated mg of amino acids per 20 ml[a]			
	0	0.5	5	15
	mg	mg	mg	mg
None	59.3			
DL-α-Alanine		49.3	58.8	68.3
L-Arginine-HCl		56.3	64.8	71.3
α-Amino *n*-butyric acid		41.3	46.5	43.5
DL-Aspartic acid[b]		45.3	52.0	75.0
β-Alanine		15.3	2.0	1.0
L-Cystine		47.5	9.0	4.0
L-Glutamic acid		46.3	50.0	66.0
Glycine		49.8	60.0	70.8
L-Histidine-HCl		53.5	51.8	48.0
L-Hydroxyproline		48.3	25.3	25.8
DL-Isoleucine		45.8	52.0	50.0
L-Leucine		45.8	41.8	47.5
L-Lysine-HCl		50.0	49.5	52.8
DL-Methionine		42.5	30.5	34.3
L-Monosodium glutamate		47.0	52.0	60.3
DL-Norleucine		32.5	28.0	28.3
DL-Norvaline		41.0	37.8	43.0
DL-Ornithine		51.3	51.0	58.5
DL-Phenylalanine		44.8	38.5	34.5
L-Proline		47.3	57.0	71.8
DL-Serine		48.0	58.5	57.3
DL-Threonine		47.0	49.0	54.5
DL-Tryptophane		40.0	38.5	30.8
L-Tyrosine		46.8	41.0	50.8
DL-Valine		39.8	40.0	46.3

[a] The figures given are means of 2 experiments. Differences of 4.4 are significant at 5 per cent level; differences of 5.8 are significant at 1 per cent level.
[b] Adjusted to pH 4 with NaOH.

TABLE 2.—*Growth of Alternaria solani on basal medium containing growth-stimulating amino acids, alone and in combination*

Amino acids added and amounts added per 20 ml	Dry weight after 72 hours
	mg
None	41.0[a]
5 mg L-Arginine-HCl	49.0
20 mg L-Monosodium glutamate	60.0
5 mg L-Arginine + 20 mg L-monosodium glutamate	45.0
None	43.5[c]
5 mg DL-Aspartic acid[b]	54.2
15 mg L-Proline	54.8
5 mg DL-Aspartic acid[b] + 15 mg L-proline	74.2
None	47.5[d]
5 mg DL-Aspartic acid[b]	61.5
5 mg L-Arginine-HCl	53.0
5 mg DL-Aspartic acid[b] + 5 mg L-arginine-HCl	75.5
None	34.5[e]
15 mg DL-Aspartic acid[b]	69.0
15 mg L-Arginine-HCl	44.5
15 mg DL-Aspartic acid[b] + 15 mg L-arginine-HCl	90.0

[a] The growth weights are means of 2 experiments. Differences of 4.5 are significant at the 5 per cent level; differences of 6.6 are significant at the 1 per cent level.
[b] Aspartic acid was adjusted to pH 4 with NaOH.
[c] The growth weights are means of 3 experiments. Differences of 4.5 are significant at the 5 per cent level; differences of 6.3 are significant at the 1 per cent level.
[d] Differences of 6.1 are significant at the 5 per cent level; differences of 10.2 are significant at the 1 per cent level.
[e] Differences of 12.2 are significant at the 5 per cent level; differences of 20.2 are significant at the 1 per cent level.

Aspartic acid plus proline had an additive effect (Table 2); in 2 other experiments in which aspartic acid was used at the 1-mg or 2-mg levels, there was always an additive effect when these 2 amino acids were used together.

When used at certain concentrations, aspartic acid and arginine in combination had additive effects upon growth (Table 2). In other experiments, no additive effects were obtained when the amount of arginine was 5 mg and that of aspartic acid was 1 or 2 mg.

Combined effects of 2 amino acids that inhibit growth when used alone.—Table 3 presents results of experiments designed to determine the combined effect of 2 amino acids that were strong or moderate inhibitors of growth when used alone. When 2 growth-inhibiting amino acids act together, their inhibitions may be additive, e.g. α-amino *n*-butyric acid and hydroxyproline. Although constantly looked for in all experiments, this kind of result was rare. A partial removal of inhibition was a more common response, e.g. β-alanine and α-amino *n*-butyric acid at 2 and 0.5 mg/20 ml, respectively. Occasionally, 2 inhibitors together will enhance growth above the level occurring in their absence, as with β-alanine and α-amino *n*-butyric acid at 2 and 5 mg/20 ml, respectively. Sometimes the combined effect is no different from the effect of the stronger inhibitor, as was the case with β-alanine and hydroxyproline at 2 and 0.5–5 mg/20 ml, respectively.

The effects of single amino acids on the inhibitions due to β-alanine and to cystine.—Since β-alanine and cystine were the strongest and most consistant inhibitors of this organism, the effects of single amino acids on these inhibitions were tested. The essential results are given in Tables 4 and 5.

Four loosely delimited types of effect are evident: no change of inhibition, partial removal of inhibition, complete removal of inhibition, and better growth than in the absence of amino acids. Arginine falls into the first group, whereas alanine and glycine (at its highest concentration) fall into the last group when used with either β-alanine or cystine.

Interactions between different concentrations of proline, which strongly enhances growth when alone, and β-alanine, which strongly inhibits growth when alone.—The results in Table 6 demonstrate clearly that an amino acid that has 1 kind of effect in 1 nutritional environment can have the opposite effect in another. The positive effect of β-alanine was pronounced only at the higher concentrations of proline.

The effects of β-alanine and cystine in the absence of vitamins.—The basal medium in all experiments so

TABLE 3.—*Growth of Alternaria solani on basal medium containing various amounts of growth-inhibiting amino acids, alone and in combination*

Amino acids added	Amount added (mg/20 ml)	Increase in dry weight in 72 hours on basal medium plus indicated amounts of amino acids (mg/20 ml)						
		β-Alanine			L-Hydroxyproline			
		0	0.5	2	0	1	2.5	5
		mg	mg	mg	mg	mg	mg	mg
α-Amino *n*-butyric acid [a]	0	33.0		2.0	41.5	34.5	28.0	27.0
	0.5	25.0		15.5	34.0	21.5	14.0	9.0
	5	24.5		40.5				
	15	25.5		32.0				
L-Hydroxyproline[b]	0	36.0		6.0				
	0.5	22.5		4.0				
	5	18.0		6.5				
	15	28.0		11.0				
L-Cystine[c]	0	44.8	18.0	3.8	40.5			14.5
	1	43.0	19.0	6.0				
	3	14.8	20.5	6.5	1.0			1.0

[a] Results with α-amino *n*-butyric acid and β-alanine, omitting the result arising from the absence of both substances, are significantly different at the 5 per cent level if the difference is as great as 7.6; at 1 per cent level if 11.2. For α-amino n-butyric acid and L-hydroxyproline, these differences are 5.8 and 8.7, respectively.

[b] Results with L-hydroxyproline and β-alanine, omitting the results arising from the absence of both amino acids, are significantly different at the 5 per cent level if the difference is as great as 4.6; at 1 per cent level if 6.9.

[c] Results with L-cystine and β-alanine, omitting the result arising from the absence of both amino acids, are significantly different at the 5 per cent level if the difference is 3.3; at 1 per cent level if 4.6 . For L-cystine and L-hydroxyproline, these differences are 5.7 and 9.5, respectively.

far reported in this paper contained 3 vitamins: biotin, choline, and thiamine. These vitamins, although not essential factors, greatly increase the growth rate of this fungus.[3] The experiment reported in Table 7 was

TABLE 4.—*Growth of Alternaria solani on basal medium containing growth-inhibiting β-alanine (2 mg/20 ml) in combination with various amino acids*

Amino acid added	Increase in dry weight in 72 hours on basal medium plus indicated mg of amino acids (mg/20 ml)[a]			
	0	0.5	5	15
	mg	mg	mg	mg
Control, basal medium only	41			
Control, basal medium plus 2 mg/20 ml β-alanine	7			
β-Alanine (2 mg/20 ml) plus:				
DL-α-Alanine		13.3	41.0	55.8
L-Arginine-HCl		7.8	9.3	10.8
α-Amino *n*-butyric acid		25.5	47.5	34.8
Glycine		13.5	24.5	56.0
L-Histidine-HCl		10.5	12.5	8.5
L-Hydroxyproline		4.0	8.8	18.3
DL-Isoleucine		10.0	38.5	39.8
L-Leucine		30.5	46.5	55.5
L-Lysine-HCl		4.5	5.3	6.3
DL-Methionine		23.3	29.5	38.0
DL-Norleucine		11.3	37.0	29.0
DL-Norvaline		30.8	43.8	49.0
DL-Ornithine		9.3	8.3	7.8
DL-Phenylalanine		9.0	37.3	49.0
L-Proline		22.0	53.8	65.5
DL-Serine		10.0	23.5	43.3
DL-Threonine		7.5	42.5	46.0
DL-Tryptophane		12.5	13.5	14.5
L-Tyrosine		11.3	14.3	17.3
DL-Valine		13.0	33.5	45.3

[a] Differences of 2.8 are significant at the 5 per cent level; differences of 3.7 are significant at the 1 per cent level.

TABLE 5.—*Growth of Alternaria solani on basal medium containing growth-inhibiting cystine (3 mg/20 ml) in combination with various amino acids*

Amino acid added	Increase in dry weight in 72 hours on basal medium plus indicated amounts of amino acids (mg/20 ml)[a]			
	0	0.5	5	15
	mg	mg	mg	mg
Control, basal medium only	43.8			
Control, basal medium plus 3 mg/20 ml of L-cystine	1.0			
L-Cystine (3 mg/20 ml) plus:				
DL-α-Alanine		24.0	46.8	55.0
L-Arginine-HCl		2.0	3.5	3.8
α-Amino *n*-butyric acid		25.8	42.0	38.2
Glycine		13.8	48.3	59.7
L-Histidine-HCl		10.8	17.7	20.8
L-Hydroxyproline		8.5	7.2	12.2
DL-Isoleucine		15.8	20.5	27.3
L-Leucine		22.3	32.0	37.7
L-Lysine-HCl		16.5	19.2	27.3
DL-Methionine		7.5	20.8	25.3
DL-Norleucine		11.7	19.2	27.8
DL-Norvaline		14.3	25.8	28.3
DL-Ornithine		7.3	9.2	17.2
DL-Phenylalanine		14.8	28.8	32.3
L-Proline		16.5	18.0	27.2
DL-Serine		11.5	33.0	47.5
DL-Threonine		11.5	27.3	37.5
DL-Tryptophane		8.2	7.3	8.7
L-Tyrosine		7.7	13.7	27.8
DL-Valine		9.7	17.0	31.7

[a] The figures given are means of 3 experiments. Differences of 4.9 are significant at the 5 per cent level; differences of 6.5 are significant at the 1 per cent level.

TABLE 6.—*Growth of Alternaria solani on basal medium containing various combinations of growth-stimulating proline and growth-inhibiting β-alanine*

Amount of L-proline added (mg/20 ml)	Increase in dry weight in 72 hours on basal medium containing indicated amounts of β-alanine (mg/20 ml)[a]				
	0	0.5	1	2	4
	mg	mg	mg	mg	mg
0	45.5	7.5	1.5	1.0	1.0
10	70.5	73.0	74.5	73.0	68.5
15	76.5	88.5	88.0	82.5	78.0
20	78.0	96.0	94.0	92.5	90.5

[a] Differences of 4.8 are significant at the 5 per cent level; differences of 6.5 are significant at the 1 per cent level.

designed to determine if the inhibiting amino acids, β-alanine and cystine, would inhibit more strongly in the absence of 1 or more of these vitamins than in their presence. Since preliminary experiments had partly established that some strong inhibitions could be expected, these experiments were planned so growth could be measured at 6 days as well as at 3 days, the time used for all the preceding experiments.

In general, both β-alanine and cystine inhibited more strongly in the absence of vitamins than in their presence (Table 7).

DISCUSSION.—During the preliminary experiments that led to those reported in this paper, the "balance hypothesis of parasitism"[4] appeared to be a working hypothesis that might guide experimentation and give meaning to the facts from nutrition experiments in terms of the host-parasite relationship. Stated in a general way, this hypothesis assumes that host specificity and related problems are explicable in terms of the effect of the host's normal diffusible metabolites upon the parasite. If such is the case, only by knowing in intimate detail the metabolites of the host available to a parasite and the response of the potential parasite to the combination of substances supplied by the host will one be able to get a basic view of the host-parasite relationship.

Since direct evidence for or against this view will be exceedingly difficult or impossible to get, it may be necessary to test the hypothesis on the basis of circumstantial evidence. A number of consequences related to circumstantial evidence were mentioned in the outline of the hypothesis.[4] One of these, which concerns the "complex nutrition" of the parasite, is enlarged upon here and examined in relation to the facts reported.

Most potential hosts, plant or animal, are immune to attack by most parasites. All hosts supply to their parasites (and to potenial parasites that can come in contact with the host's moist inner cell walls or cell membranes) a complex nutritional environment. This environment contains many organic and inorganic foods that are the diffusible metabolites from the host cells. If this nutritional environment determines whether a parasite will or will not grow, one would expect that most of the complex synthetic media would inhibit growth of the parasite and that only a few synthetic media would support growth. The results of the above experiments seem to argue against this consequence. Only a few combinations of nutrients produced strong inhibitions, although inhibiting combinations were constantly sought. The results of tests with growth-inhibiting amino acids in the absence of vitamins (Table 7), however, suggest that possibly the right combinations of nutrients were not tried.

The conclusion that the present data are inconsistent with the balance hypothesis may not be valid because of a number of possibilities: 1) the media used were extremely simple when compared to the putative nutri-

[4] Lewis, R. W. 1953. An outline of the balance hypothesis of parasitism. Am. Naturalist **87**: 273-281.

TABLE 7.—*Growth of Alternaria solani on a medium containing inorganic foods, glucose, and growth-inhibiting amino acids, with and without vitamins*

Vitamins added[a]	Increase in dry weight in 3 and 6 days on media containing indicated amino acids[b]					
	None		β-Alanine		L-Cystine	
	3 Days	6 Days	3 Days	6 Days	3 Days	6 Days
	mg	mg	mg	mg	mg	mg
None	5.0	52.7	2.8	43.7	0.3	22.2
D-Biotin	13.1	70.0	5.3	39.8	0.7	16.8
Choline chloride	8.5	75.0	7.5	70.8	1.0	43.3
Thiamine-HCl	6.8	77.5	4.7	49.8	2.2	45.3
D-Biotin + choline chloride	26.7	112.2	9.0	82.7	8.0	71.3
D-Biotin + thiamine-HCl	26.2	110.5	5.3	46.2	3.8	52.5
Choline chloride + thiamine HCl	10.5	84.8	1.3	83.3	4.3	82.8
D-Biotin + choline chloride + thiamine HCl	50.2	141.3	9.0	113.8	23.7	90.7

[a] Amounts added were as follows: D-biotin, 0.0001 mg/20 ml; choline chloride, 0.04 mg/20 ml; thiamine-HCL, 0.02 mg/20 ml.

[b] Amounts added were as follows: β-alanine, 2 mg/20 ml; L-cystine, 3 mg/20 ml. The growth weights are means of 3 experiments. For the results after 3 days' growth, differences of 2.5 are significant at the 5 per cent level; differences of 3.4 at the 1 per cent level. For the results after 6 days' growth, differences of 13.6 are significant at the 5 per cent level; differences of 18.1 at the 1 per cent level.

tional environment supplied by the host; 2) the inorganic foods chosen may have been very different from those encountered in the host; 3) a less freely saprophytic parasite might have given results closer to prediction; and 4) the physical environment in culture was far from being comparable to the environment supplied by the host. Any one of these could possibly account for the disagreement between the facts and the prediction, but since each objection is testable, they point clearly to future experimentation.

The experience and knowledge derived from the above experiments strongly suggest that it will be better in the future to make thorough studies of the chemical content of the host before spending much time on nutrition experiments with the parasite. All one needs to know at first is that the parasite will grow on synthetic media. Then when analyses of the host have supplied considerable information about the diffusible substances that are the probable foods of the parasite, nutrition experiments can be designed that begin to approximate the "definitive nutrition"[4] of the parasite, i.e. the nutrition supplied by the host to the parasite. A minimum analysis of the host should be a qualitative and quantitative estimate of the free inorganic substances, amino acids, organic acids, sugars, and vitamins. How exact the analyses must be and how far beyond these categories one must go cannot be said at present.

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ALTERING DISEASE RESISTANCE WITH IONIZING RADIATION AND GROWTH SUBSTANCES[1]

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SUMMARY

In comparative studies of the effects of X-radiation and growth substances upon resistance to Fusarium wilt of tomato, shoot irradiation decreased disease resistance of highly susceptible (Bonny Best), multiple-gene-resistant (Pritchard), and single-gene-resistant (Pan American) tomatoes. Root irradiation 2–4 days before inoculation increased their resistance, as also did naphthalene acetic acid. Indole acetic acid increased resistance in nonirradiated plants and largely prevented any loss of resistance 1) due to whole plant irradiation at inoculation time or 2) due to treatment with maleic hydrazide. Exposure of plants to solutions of metabolic inhibitors before and after root irradiation did not prevent the appearance of resistance. Shoot irradiation decreased the resistance of the stem as well as of the shielded roots. Cuttings from irradiated roots and plants only part of whose roots had been irradiated were not made resistant. Resistance produced by root irradiation or treatment with naphthalene acetic acid reached a maximum after 2–8 days and then disappeared; these time changes in resistance were dependent upon temperature. The decreases in resistance caused by shoot irradition and by maleic hydrazide also changed with time.

Both ionizing radiation and growth substances produce certain effects in common. For example, they both alter resistance to Fusarium wilt of tomato (3, 16). In addition, ionizing radiation decreases the level of auxin (13). For these reasons, comparative studies of the effects of ionizing radiation and growth substances upon resistance were made and are reported here.

In this study, the response to radiation and to growth substances of 3 tomato varieties was determined. Also, attempts were made to inhibit the effect of radiation. Finally, the translocation and time and temperature changes of the effects were determined and compared.

Materials and methods.—The 3 varieties of tomato employed were Bonny Best, which is highly susceptible to Fusarium wilt; Pritchard, which has multiple-gene resistance; and Pan American, which has single-gene resistance (1). Plants grown in sand culture to the 4- to 6-leaf stage were uprooted and irradiated by exposing them on wet cotton to soft X-rays at 5r/sec. (roentgen/second) under conditions described earlier (16). When portions of plants were irradiated, the rest of the plant was shielded by lead during exposure; the checks were exposed in an adjacent room. Growth substances were applied by spraying or dipping the foliage in aqueous solutions. Plants were inoculated by dipping the roots in suspensions of bud cells from shake cultures of *Fusarium*

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oxysporum f. *lycopersici*. (Sacc.) Snyd. & Hans. Bonny Best and Pritchard were inoculated with the R5–6 strain of the fungus whereas Pan American, which is immune to this strain, was inoculated with race 2 furnished by Dr. L. J. Alexander. Vascular symptoms were scored after about 2 weeks' incubation at 27°C. To express disease severity quantitatively in terms of vascular discoloration, the mean number of the 6 possible vascular bundles discolored per internode was used. This grade ranges from 0 to 6 and is called "vascular index." More detailed descriptions of methods have already been published (4, 16).

VARIETAL RESPONSE TO RADIATION.—If radiation and growth substances affect the resistance of tomato plants to wilt by means of a common mechanism, both treatments should alter the resistance of susceptible and resistant varieties of tomato similarly.

Roots or shoots of plants were exposed to 0 or 18 Kr (10^3 roentgens) and were inoculated 4 days later. When shoots were irradiated, roots of Bonny Best and Pritchard tomatoes were stunted and necrotic but were normal when the roots themselves received treatment. Plants with irradiated roots were taller than the checks at the end of the incubation period. Irradiation of Bonny Best tomato roots produced a large increase in resistance, whereas shoot exposure caused a small decrease in resistance; irradiation of Pritchard roots led to a small increase and shoot treatment to a large decrease in resistance (Table 1). These results were confirmed by a second experiment under the same conditions as well as one run at 18° with an extended incubation period.

Exposure of Pan American shoots to 18 Kr on the day of inoculation increased the vascular index by 0.54 from one of 2.54 for the check; the increase was not statistically significant but was in the same direction as for other varieties. Exposure of the roots to 18 Kr 2 days before inoculation decreased the vascular index by 1.61 from one for the check of 2.56; the decrease was statistically significant at the 10 per cent level and in the same direction as for the other varieties.

A further similarity of Bonny Best and the resistant Pritchard variety was demonstrated. Roots of both varieties were exposed to 9 Kr X-rays and then inoculated after 0 and 2 days. Radiation caused no change in vascular index in either variety when inoculation immediately followed irradiation. When inoculation was delayed 2 days, however, the vascular index was decreased significantly ($P = 0.05$) in both varieties (Table 2).

TABLE 1.—*Vascular index (V.I.)[a] in 2 varieties of tomatoes exposed to 18 Kr rays 4 days before inoculation with Fusarium oxysporum f. lycopersici*

Variety	Change in V.I. following indicated treatment			
	Check V.I.	Root irradiation	Shoot irradiation	LSD (P=0.05)
Bonny Best	4.05	−1.40	+1.57	1.08
Pritchard	2.30	−0.93	+2.54	1.32

[a] Vascular index is the mean number of the 6 possible vascular bundles discolored per internode; the index ranges from 0 to 6.

TABLE 2.—*Vascular index[a] in 2 varieties of tomatoes inoculated with Fusarium oxysporum f. lycopersici 0 and 2 days after irradiation of roots*

Radiation dose (Kr)	Vascular index[b] in plants inoculated at the stated intervals following irradiation			
	0 Days		2 Days	
	Bonny Best	Pritchard	Bonny Best	Pritchard
0	3.2	2.4	3.0	2.8
9	3.3	2.2	1.1	0

[a] Vascular index is the mean number of the 6 possible vascular bundles discolored per internode; the index ranges from 0 to 6.
[b] LSD (P=0.05) = 1.8.

Another similarity between Bonny Best and single-gene resistant Pan American was shown. Naphthalene acetic acid (NAA) and other growth substances applied before inoculation increase the resistance of Bonny Best (3). When the foliage of Pan American tomatoes was dipped in 2.7×10^{-4} M NAA 2 days before inoculation with race 2 of *F. oxysporum* f. *lycopersici*, the vascular index was reduced from 2.6 in the check to 0 in the treated plants. The LSD ($P = 0.05$) was 2.1.

Irradiation of 3 varieties produced similar effects upon their resistance to pathogenic races of Fusarium. Treatment with growth substances produced similar increases in resistance on highly susceptible and highly resistant varieties. These things occurred despite wide differences in the source, level, and genetic characteristics of the varieties' resistance. In contrast, shoot and root irradiation produced not only different (16) but opposite effects upon resistance in all 3 varieties.

ATTEMPTED INHIBITION OF THE EFFECT OF RADIATION.—Two mechanisms by which radiation changes resistance are suggested. First, radiation may alter resistance by changing the level of growth substance in the plant. A second mechanism, which would not exclude the first, is that alteration may be a process dependent upon respiration. Studies of inhibition should test these hypotheses.

Attempts to inhibit by means of growth substances have taken 2 forms. First, whole Bonny Best plants were exposed to 0, 9, and 27 Kr and then dipped in 0, 0.6, and 2.8×10^{-3} M indole acetic acid (IAA) at inoculation time. Radiation in this manner caused a statistically significant decrease in resistance as was expected; IAA increased resistance in the nonirradiated plants and prevented any decrease due to radiation. Similar results were obtained in a second experiment.

A second design was employed. Here Bonny Best plants were subjected to 0, 5, and 10 Kr. Plants were sprayed with 0, 0.5, and 1.0×10^{-3} M IAA at inoculation time as well as 7 and 14 days later. Radiation increased defoliation, an effect prevented by IAA.

Maleic hydrazide (MH), a plant-stunting agent whose characteristics have been reviewed by Zukel (17), decreases wilt resistance if applied to tomatoes at inoculation time (14). Antiauxinic (10) and effects similar to radiation (15) have been ascribed to MH. Therefore, MH and radiation were compared by attempting to inhibit with IAA the decrease in resistance induced by MH.

Bonny Best plants that had been dipped in 0 and 2.7×10^{-3} M MH were also dipped in 0, 0.6, and 2.8×10^{-3} M IAA. MH applied in this manner caused a significant decrease in resistance as was expected; IAA increased resistance in plants not treated with MH and largely prevented the decrease caused by MH. Similarly, sprays of 0.5 and 1.0×10^{-3} M IAA applied in another experiment 0, 7, and 14 days after inoculation prevented the increase in defoliation caused by MH applied at inoculation time. Thus, the decreases in resistance generally caused by radiation or MH applied at inoculation time did not appear when IAA was also applied.

Next, a variety of metabolic inhibitors was employed to test whether the increase in resistance following root irradiation was dependent upon a physiological, energy-consuming process. Inhibitors of this type can prevent the manifestation of inherited resistance (5). Small Bonny Best seedlings were stood in water and in the following water solutions: water, 10^{-3} M KCN, 0.5×10^{-3} M sodium diethyl dithiocarbamate, 10^{-3} M *p*-chloromercuribenzoate, and 10^{-5} M 2,4-dinitrophenol. Solutions were renewed daily. On the third day, roots were exposed to 9 Kr. On the fifth day, the plants were removed from the solution, the roots washed in running water, and the plants inoculated by dipping the roots in Fusarium bud cells.

Radiation caused an increase in resistance, whether the roots had been standing in water, KCN, sodium diethyl dithiocarbamate, or 2,4-dinitrophenol. Evidently resistance caused by radiation is qualitatively different from inherited resistance. The *p*-chloromercuribenzoate produced a peculiar result: inoculation of all plants standing in its failed whether irradiated or not. Although we cannot say whether or not it prevented the appearance of resistance following irradiation, it is a prospective chemotherapeutant.

TRANSLOCATABILITY OF EFFECTS PRODUCED BY RADIATION.—If radiation alters resistance by affecting the level of some substance in the plant, e.g. growth substance, then irradiation of one part of the plant will affect resistance elsewhere. If effects elsewhere are not observed, then this hypothesis is untenable; if they are observed elsewhere this hypothesis or an alternative is true. We shall consider shoot and root irradiation separately.

Shoot irradiation affects the susceptibility of the exposed stem as well as the shielded root. This was demonstrated by exposing the shoots of Pritchard tomatoes to 18 Kr and then inoculating them 3 days later by dipping roots into inoculum, inserting into the stem 2 toothpicks overgrown with Fusarium or by standing stem cuttings in washed bud cells (8). The cuttings were shaded throughout the period during which they were in the bud-cell suspension, the third through the seventh day following irradiation, and were then rooted by being placed in wet sand. The vascular index was significantly increased by shoot irradiation, an increase that occurred regardless of whether roots or shoots were inoculated (Table 3). These observations are consistent with the hypothesis that some mobile agent that controls resistance is affected by shoot irradiation.

Next, the question of whether root irradiation increases resistance by affecting a mobile substance was studied. Several designs were employed. In 1 experiment, the roots of Bonny Best tomato plants were exposed to 12 Kr. Four days later the roots were removed and the cuttings inoculated by standing them in washed bud cells. No significant decrease in vascular index was produced by radiation in this experiment; no decreases occurred in 2 other experiments of similar design. That is, irradiated plants were no more resistant than the checks when the stems were inoculated thus.

Second, only 1 side of the roots or only scattered areas of the roots were exposed to 18 Kr. This was accomplished by use of pie-shaped or perforated shields during irradiation. Four days later the plants were inoculated by the usual root-dip method. When only part of the plants' roots were irradiated, they were no more resistant than the checks.

Finally, plant roots were exposed to 18 Kr in the usual manner, but the plants were then set 1 cm deeper in the sand than before. This caused new roots to form from the nonirridated stem. Four days later, the old roots were cut away and the new roots inoculated by dipping them in Fusarium bud cells. Irradiated plants thus inoculated through nonirradiated roots were no more resistant than the corresponding checks. Thus, root irradiation has in no case caused the stem or nonirradiated roots to become resistant after the irradiated roots were removed.

TIME CHANGE IN INDUCED RESISTANCE.—Information concerning the time change in resistance following irradiation or treatment with growth regulators is valuable for 3 reasons. First, information on the time required to attain maximum resistance following treatment is important in designing experiments. Second,

TABLE 3.—*Vascular index*[a] *in tomatoes following 3 methods of inoculation with Fusarium oxysporum f. lycopersici*

Shoot irradiation (Kr)	Index following indicated method of inoculation			
	Root[b]	Toothpick	Cutting	Mean[c]
0	2.0	3.1	1.5	2.2
18	2.9	5.3	2.0	3.4

[a] Vascular index is the mean number of the 6 possible vascular bundles discolored per internode; the index ranges from 0 to 6.
[b] LSD (P=0.05) = 1.9 (means of 10).
[c] LSD (P=0.05) = 1.1 (means of 30).

duration of the increase will determine any practical use of the method. Finally, the manner in which the increase changes could give a clue as to its nature, particularly if it is dependent upon temperature.

The time changes in resistance following root irradiation or treatment with NAA were determined at 2 temperatures, 18 and 27°C. Bonny Best plants were sprayed with 0.27×10^{-3} M NAA, their roots were exposed to 9 Kr, or they served as checks and were uprooted, inoculated, and incubated at the same time and temperature as the treated plants. The plants were all grown at 27° until they were irradiated or sprayed. Then half of the plants were incubated at 18° and half at 27° until they were inoculated. Following inoculation, all plants were incubated at 27°. Thus all plants were held at a temperature of 27° except for the period during which the plants may become resistant, the period between treatment and inoculation.

The time course of change in resistance was measured in 2 experiments of 5 replicates each. The mean vascular index for each treatment and experiment was expressed as a percentage of the index for the corresponding check; these relative vascular indices for each of the 2 experiments are shown (Fig. 1, 2) with a bar connecting corresponding values in the 2 experiments. The length of the bar gives a measure of reproducibility from experiment to experiment. The "days delay" along the abscissa is the time from treatment to inoculation, the time allowed for the increase

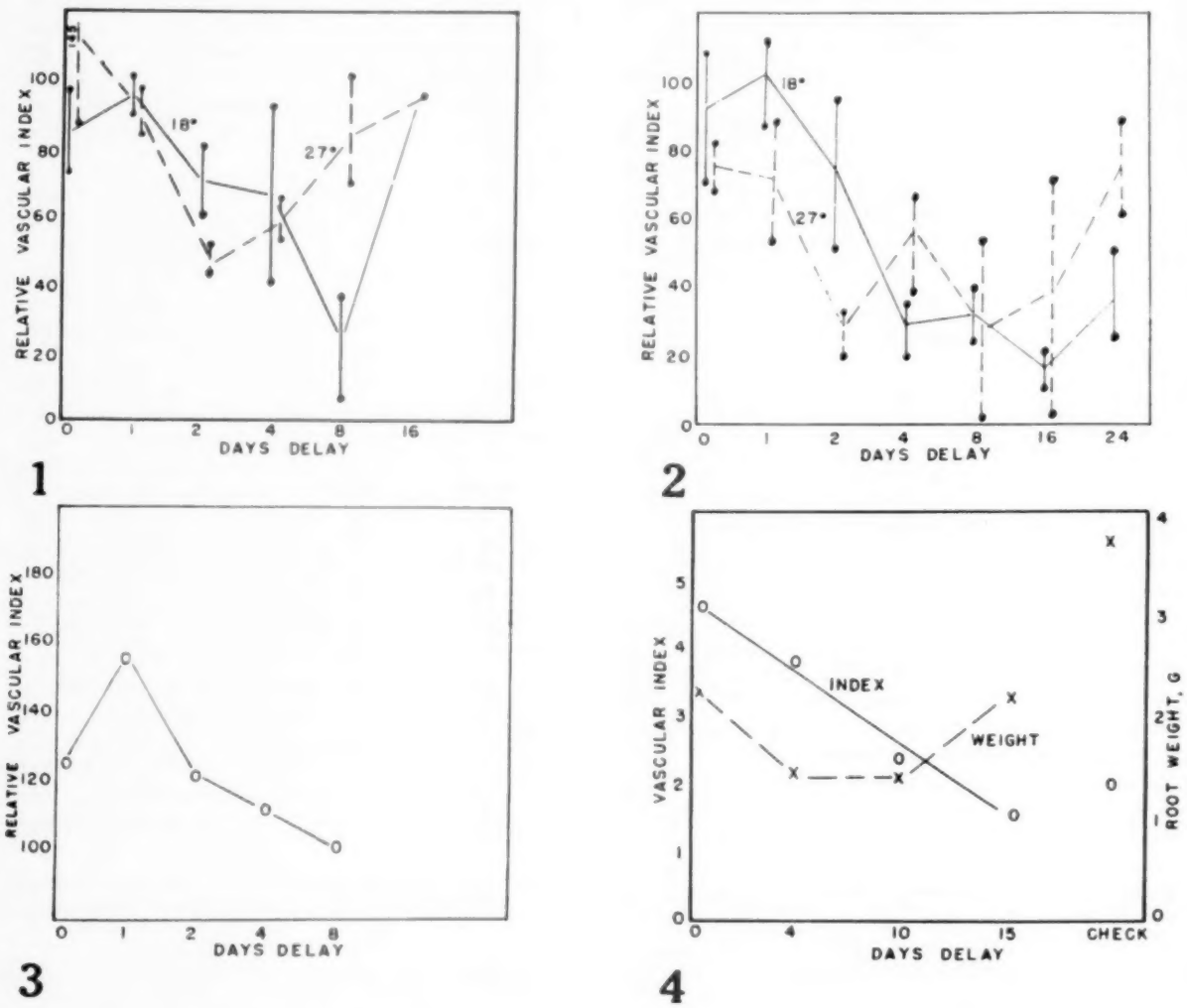

FIG. 1-4.—Fig. 1. Relative vascular index of plants whose roots were exposed to 9 Kr and then inoculated following various delays. Relative index is expressed as percentage of a check. Vertical bars extend from mean relative index for one experiment to the mean for a second experiment.—Fig. 2. Relative vascular index of plants treated with 2.7×10^{-4} M napthalene acetic acid. Inoculation was delayed, index expressed, and means shown as in Figure 1.—Fig. 3. Relative vascular index of plants whose shoots were exposed to 18 Kr. Inoculation was delayed and index expressed as in Figure 1.—Fig. 4. Vascular index of plants treated with 2.7×10^{-3} M maleic hydrazide at stated times before inoculation. Check was unsprayed.

in resistance to appear and disappear at 18° or 27°.

At 27°, the maximum increase in resistance, as shown by minimum relative index, appeared 2 days after root irradiation (Fig. 1) or NAA spray (Fig. 2). The effects of the 2 agents differed, however, in their rates of disappearance: the increase produced by NAA lasted at least 16 days whereas the one produced by radiation had disappeared after 8 days at 27°. At 18°, both the appearance and disappearance of resistance were delayed.

The time change of resistance following shoot irradiation of Pritchard tomatoes was determined at 27°. Shoots were exposed to 18 Kr. Then, following 0–8 days delay, the plants were inoculated. The mean vascular indices for 5 replicates are presented as percentages of the check indices (Fig. 3). The maximum decrease in resistance, as shown by a maximum relative index, occurred shortly after irradiation, and the decrease disappeared after 4–8 days.

The time change in resistance following treatment with MH was determined, because MH has already been shown to produce certain changes in common with radiation (14, 15). Bonny Best tomato plants were sprayed to runoff with 2.7×10^{-3} M MH 15, 10, 4, and 0 days before inoculation. Check plants were not sprayed. The vascular index was significantly increased by treatment at inoculation time as was expected (14); that is, resistance was decreased (Fig. 4). As treatment was advanced up to 15 days ahead of inoculation time, the decrease in resistance grew smaller. Roots, in particular, are stunted by MH (6); however, the stunting of roots was not related in any simple manner to the change in resistance (Fig. 4). Thus, MH does not always cause increased susceptibility, because the change depends upon the period between time of application and inoculation.

Discussion.—The practical usefulness of at least 1 of the methods studied, treatment of tomato seedlings with growth substance to render them resistant to Fusarium for 2–4 weeks, seems worth testing on a field scale in regions where the disease is a problem. The additional resistance this treatment bestows upon plants with inherited resistance adds to its potentiality. Also, this treatment has increased resistance to Verticillium wilt of tomato in experiments in our greenhouse. If resistance to wilt fungi at the time of transplanting and consequent wounding is important, then this method should provide an effective wilt control. Irradiation of seedlings probably would not be a successful practical control measure, because the plants are made more resistant for only about 1 week.

The observation that irradiation of root and shoot have opposite effects bears upon the question of localization of resistance first raised by May's grafting experiments (11). Opinions have varied as to what each organ does to its opposite number relative to resistance. Different workers have maintained that resistance is localized in the roots of resistant varieties (7), is present in both roots and shoots of resistant varieties (12), or is present in the roots of resistant varieties and the stems of all varieties (9). The radiation experiments permit an examination of the phenomenon in intact plants.

The grafting experiments of Heinze and Andrus (7) can be represented thus:

$$\frac{B}{b} \rightarrow 0 \qquad \frac{P}{p} \rightarrow 1 \qquad \frac{P}{b} \rightarrow 0 \qquad \frac{B}{p} \rightarrow 1$$

where large letters refer to contributions to resistance by Bonny Best and Pan American shoots, small letters to contributions by roots, and 0 and 1 to susceptible and resistant plants, respectively. Scheffer and Walker's (12) experiments with cuttings can be added:

$$\frac{B}{\text{later } b} \rightarrow 0 \qquad \frac{P}{\text{later } p} \rightarrow 1$$

Our results with radiation applied prior to inoculation can be added if we assume that radiation is destructive and removes something as indicated by a minus sign:

$$\frac{-B}{b} \rightarrow -\tfrac{1}{2} \qquad \frac{B}{-b} \rightarrow \tfrac{1}{2}$$

That is, shoot irradiation decreases what little resistance Bonny Best has, root irradiation increases it. All of these hypotheses and results are consistent with the production of an inhibitor of resistance by roots, especially Bonny Best, and of an increase of resistance by shoots. The preceding studies on the translocatability of radiation-induced resistance also are consistent with this hypothesis.

Whole plant irradiation $\left(\frac{-B}{-b}\right)$ leads to decreased resistance, i.e. $-\frac{1}{2}$, if inoculation occurs immediately. If inoculation is delayed, the plant becomes resistant, i.e. $+\frac{1}{2}$ (16). This is perhaps due to a recovery in B, perhaps to a further degeneration in b.

The time changes in resistance following root and shoot irradiation (Fig. 1, 3) can also be explained in terms of the decay and eventual recovery of both the inhibitors and increasers of resistance. These processes, to reason from their temperature dependence and appearance in a variety of plants, must be common biological processes; however, our failure to inhibit them by a number of metabolic inhibitors leaves us without any knowledge of their particular nature.

A consideration of the time changes in resistance following treatment with root irradiation or NAA reveals a paradox: inoculation 2 days after treatment leads in 14 days to only one-half the disease severity of the check; nevertheless, inoculation at treatment time only 2 days before leads to the same severity as the check although for 12/14 of the incubation period this susceptible host is presumably in the same condition as the resistant one inoculated only 2 days later. This paradox can be explained if one assumes that inoculation at treatment time prevents the production of resistance during the ensuing 2 days; then for 12/14 of the incubation period the host inoculated 0 days following treatment would not be like the one

inoculated on day 2.

The well-known decrease in natural growth substances in irradiated plants (13) and the similarity of action of irradiation and growth substances has already led to the suggestion that radiation alters resistance by altering growth-substance level (16). The concept of a radiation-sensitive increaser of resistance in the shoots and inhibitor in the roots was introduced above. Obviously, the increaser could be synthesis of growth substance and the inhibitor could be growth substance inactivation if we accept the classical view (2) that these substances are synthesized in the shoots and inactivated in the roots.

More conclusive evidence that radiation alters resistance by altering growth substance level would be provided by inhibitor studies. As described above, treatment of plants with IAA resulted subsequently in some success in preventing decreases in resistance caused by radiation. IAA, however, increases the resistance of nonirradiated plants as well; therefore, we may be observing independent and opposite actions and not inhibition of the radiation effect.

Some variations exist, but the preceding comparisons show that root radiation belongs to a large group of agents, both physical and chemical, that alter wilt resistance in a peculiar way: treatment shortly before inoculation increases resistance more than treatment either after inoculation or long before. Shoot irradiation and at least 1 other agent, MH, have the additional property of decreasing resistance if treatment occurs near inoculation time.

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FUNGI ASSOCIATED WITH THE FLOWERS, PODS, AND SEEDS OF SOYBEANS[1]

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SUMMARY

Studies of the fungi associated with the flowers, pods, and seeds of soybeans were made at the Delta Branch Experiment Station, Stoneville, Mississippi, from 1951 to 1955. The kinds of fungi and their relative prevalence varied with season, with location within the plant, and with varieties. *Alternaria* spp. were isolated most frequently from the pistil and stamens of unopened and opened flowers and from style and stigma remains adhering to pod tips. *Cercospora kikuchii* comprised the highest percentage of isolates from seed within pods.

Infection of the pistil and stamens was less in unopened than in progressively older flowers.

The percentage of seed infection was lowest prior to maturity and gradually increased through and after maturity. In 66 per cent of the varieties and strains sampled over a 4-year period, 100 per cent of the seed were infected by the end of the sampling period, which extended from 1–6 weeks prior to maturity to 1–6 weeks after maturity. In 45 per cent of the varieties and strains sampled, 50 per cent of the seed were infected by the date of maturity. In only 1 variety (CNS) were 100 per cent of the seed infected prior to maturity. In general, infection of 100 per cent of the seed occurred sooner in early maturing varieties and strains than in later maturing ones.

It is suggested that fungi gain entrance to soybean seeds within unopened pods by the following means: 1) cracking of pod walls; 2) insect injuries, and 3) systemic infection.

Of 3845 unopened and opened flowers plated out over a 3-year period, fungus isolates were obtained from 281 or 7.3 per cent. Of 5248 styles and stigmas from pod tips plated out over the same period, fungus isolates were obtained from 3587 or 68.4 per cent. Of 11,800 seeds from within pods plated out over a 4-year period, fungus isolates were obtained from 5200 or 44.1 per cent.

Harvested soybean (*Glycine max* (L.) Merrill) seeds have been found to be infected with many kinds of fungi (4,5,6,7,8). Tervet (8) noted that frost injury increased the number of fungi present. Kilpatrick and Hartwig (4,5) found that poor quality seed yielded more fungi than did sound, high quality soybeans. Since all of the earlier investigations were made with harvested seed, a study was initiated at Stoneville, Mississippi, to determine the kind and relative prevalence of fungi associated with 1) the pistil and stamens of soybean flowers of different ages; 2) style and stigma remains adhering to soybean pod tips; and 3) soybean seed while still within the pods prior to harvest. Preliminary results were reported earlier (2,3).

MATERIALS AND METHODS.—Soybean flowers (Fig. 1), varying in ages from 1 day prior to opening to 2 days following opening, were obtained at daily intervals from 10–19 varieties and strains. The flowers were collected over a 4-week period to determine the approximate stage at which floral infection occurred. The pistil and stamens were removed easily as a unit (consisting of the stigma, style, ovary, anthers, and filaments) by pressing with the fingernail at the base of the ovary and gently pulling the petals with a pair of forceps. The entire reproductive unit was then transferred to a small square of cheesecloth by means of a spear-headed needle. This made it possible to sterilize and to rinse all sections for the same lengths of time. All sections were dipped momentarily in 50 per cent ethanol, then submerged in mercuric chloride (1:1000) for 1 minute, and finally rinsed 4 times in sterile distilled water. A sterile spear-headed needle was used to transfer the individual units to Petri dishes containing approximately 20 ml of potato-dextrose agar. Five units were spaced within each Petri dish, which was incubated at room temperature for a period of 7–10 days before final identification of the fungi was made.

In 1952, pods of 21 varieties and strains of soybeans, with portions of the stigma and style adhering (Fig. 2), were collected at 2-week intervals throughout the period of pod development to determine if fungi were associated with the floral remnants adhering to pod tips. Small sections (1–2 mm) of the style and stigma remains were removed from pod tips with a razor blade, surface sterilized, and plated in the same manner as the pistil and stamen units. The number of varieties used in 1953 and 1954, and the procedures followed, were essentially the same as those of 1952.

In 1951, pods of different varieties and strains, representing various maturity groups, were selected for studies on the fungi associated with seeds still within the pods. The varieties and strains used in the different years were essentially the same in number but not in identity. Pods of Wabash, Dorman, Ogden, and Roanoke were sampled in all 4 years. Approximately 25 3-seeded pods of each variety or strain were selected

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FIG. 1. Soybean flowers at different ages: A) 1 day prior to opening; B) day of opening; C) 1 day after opening; and D) 2 days after opening (×5).

at random at weekly intervals. Each pod was picked from a different plant, beginning from 1 to 6 weeks prior to maturity and continuing up to 6 weeks after maturity. Plants were considered mature when pods were dry and most of the leaves had abscised. Pods with visible external cracking were always avoided. Pods were surface sterilized for 20–30 seconds in 50 per cent ethanol, then transferred to a mercuric chloride solution (1:1000) where they remained for 1–20 minutes. The operator's forefingers and thumbs were submerged in the same solution as the pods, whereas the pair of forceps to be used for removing the seeds aseptically from the surface-sterilized pods was sterilized in a different solution of mercuric chloride.

Pods were removed from the mercuric chloride and opened by pressing with the thumbnail. The seeds were removed with sterile forceps and transferred to Petri dishes containing potato-dextrose agar (20–25 ml) to which 50 mg per l of the sodium salt of 2, 4-D acid (2,4-dichlorophenoxyacetic acid) was added (1). Growth of fungi was not inhibited by this concentration. No more than 2 seeds were removed from each pod, and each Petri dish contained 4 seeds evenly spaced. The seeds were selected from various locations within the pod, and each seed was labeled. Petri dishes containing the seeds were incubated at room temperatures, which varied from 65°-95°F. Subtransfers of fungi were made from infected seeds after 72 hours and at 24-hour intervals thereafter. Plated materials were discarded after 8–10 days.

EXPERIMENTAL RESULTS.—*Fungi isolated from flowers of different ages.*—The different kinds of fungi isolated from the pistil and stamens of flowers of different ages are listed in Table 1. *Alternaria* spp. were most prevalent, comprising on the average 65 per cent of the total isolates. Other than *Cladosporium* spp. and *Curvularia lunata*, no other fungus comprised more than 8 per cent of the total isolates. *Alternaria* spp. were more prevalent in 1955 than in 1953. The latter year was quite hot and dry during the flowering period, which suggests that fungus infection is more prevalent in wet years than in dry ones.

TABLE 1.—*Fungi isolated from the pistil and stamens of unopened and opened flowers of soybeans and their relative prevalence expressed as percentage of the total isolates, Stoneville, Mississippi, 1953-55*[a]

Fungus	Percentage of total isolates[b]		
	1953	1954	1955
Alternaria spp.	57	68	71
Cladosporium spp.	17	1	10
Curvularia lunata (Wakk.) Boed.	7	11	1
Fusarium oxysporum Schlecht.	6	3	8
Phomopsis sojae Lehm.	3	0	0
Penicillium varians G. Sm.	3	4	1
Aspergillus niger v. Tiegh.	1	3	2
Phyllosticta sp.	1	1	1
Rhizopus nigricans Ehr.	1	0	1
Fusarium roseum Lk.	1	0	0
Aspergillus niger v. Tiegh.	0	3	1
Trichoderma sp.	0	1	0
Chaetomium sp.	0	1	0
Neurospora sp.	0	0	1
Fusarium solani (Mart.) Appel & Wr.	0	0	1
Unidentified species	3	4	2

[a] The pistil and stamen unit plated on agar included the stigma, style, ovary, anthers, and filaments. Six surveys were made each year for each variety or strain. The number of varieties or strains sampled were as follows: 1953—17; 1954—16; and 1955—1. Of 3845 flowers plated out over the 3 years, fungus isolates were obtained from 281, or 7.3 per cent.

[b] Percentages calculated on the basis of total isolates for each year.

FIG. 2. Immature pods of soybeans showing persistence of style and stigma at tip (×6).

The data collected on the percentage of fungi isolated from the pistil and stamen units of different ages were quite similar in 2 of the 3 years. The reproductive units of unopened flowers yielded 3 per cent of the total isolates in all 3 years. Isolations from the pistil and stamen units of opened flowers on the day of opening, from 1-day-old units, and from 2-day-old units yielded 4, 4, and 9 per cent respectively, of the total isolates in 1953; 16, 13, and 41 per cent in 1954; and 77, 80, and 47 per cent in 1955. The percentages of infected units were lowest in unopened flowers and in flowers on the day they opened. The highest percentage of isolates was obtained from the reproductive units of 2-day-old flowers. The same trend was noted in each of the 3 years. In 1953 and 1954, the percentages were about the same whereas in 1955 the percentages were somewhat higher. Nineteen per cent of the pistil and stamen units yielded fungus growth in 1955, whereas only 5 and 4 per cent were obtained in 1953 and 1954, respectively. Many of the pistil and stamen units from 2-day-old flowers were found to have mycelial fragments growing from them prior to plating.

Fungi isolated from style and stigma remains adhering to pod tips.—Style and stigma remnants adhering to pod tips yielded a greater number of different fungi (Table 2) than did the pistil and stamens from flowers. *Alternaria* spp. comprised the greatest percentage of isolates in all 3 years, averaging 64 per cent. *Cladosporium* spp. were isolated more frequently in 1952 and 1953 than in 1954. This fungus may possibly have a lower moisture requirement than the other fungi. Other than this, no explanation can be offered. None of the other fungi comprised more than 6 per cent of the total isolates. Isolations from style and stigma remnants on pods approaching maturity yielded more fungi than were obtained from those on immature pods. *Cercospora kikuchii* and *Phomopsis sojae* were isolated most frequently from the remains on pods approaching maturity, but they

TABLE 2.—*Fungi isolated from styles and stigmas adhering to soybean pod tips and their relative prevalence expressed as percentage of the total isolates, Stoneville, Mississippi, 1952-54*[a]

Fungus	Percentage of total isolates		
	1952	1953	1954
Alternaria spp.	57	61	74
Cladosporium spp.	27	24	9
Fusarium oxysporum	5	5	3
Cercospora kikuchii (T. Matsu & Tomoyasu) Gardner	4	6	6
Phyllosticta spp.	2	1	4
Curvularia spp.[b]	2	1	2
Phomopsis sojae	1	Tr	Tr
Miscellaneous species[c]	2	2	1
Unidentified species	Tr	Tr	1

[a] Percentages represent an average of 9, 5, and 6 surveys made at 2-week intervals and involving 21, 24, and 38 varieties and strains of soybeans in 1952, 1953, and 1954 respectively. Of 5248 styles and stigmas from pod tips plated out over the 3 years, fungus isolates were obtained from 3587, or 68.4 per cent.

[b] Identified as *C. lunata* and *C. affinis* (?).

[c] *Aspergillus niger*, *Aspergillus* spp., *Colletotrichum destructivum* O'Gara, *Corynespora cassiicola* (Berk. & Curt.) Wei, *Fusarium roseum* Lk., *Penicillium varians*, *Penicillium* spp., and *Rhizopus nigricans* were isolated during 1 or more of the 3 years but none exceeded 0.5 per cent of the total isolates.

were never isolated from those on immature pods. None of the fungi isolated from style and stigma remains adhering to pod tips was consistently associated with individual varieties and strains.

Fungi isolated from soybean seeds within the pods.—A graphic summary of 4 years' data on the percentage of seed infection for 4 varieties of soybeans is presented in Figure 3. The sampling dates are average dates for the 4 years. Infection of seeds was lowest prior to maturity and gradually increased through and after maturity. Wabash (Fig. 3, A) became completely infected each year. Dorman (Fig. 3, B) and Ogden (Fig. 3, C) in 3 of the 4 years, and Roanoke (Fig. 3, D) in only 1 of the 4 years sampled. The 1954 plot of Roanoke was severely affected by drouth. Most of the early maturing varieties became 100 per cent infected during the sampling period, whereas the later maturing varieties took longer or never became completely infected during the sampling period. Forty-five per cent of the varieties and strains sampled were over 50 per cent infected when they matured, whereas more than 65 per cent of the varieties and strains were completely infected by the end of the sampling period. The number of seeds infected frequently increased 50 per cent or more from one sampling period to the next, although this was not consistent. CNS (not shown) was the only variety that became completely infected prior to maturity. In 1951, sampling was continued for Wabash and Ogden after the maximum infection was reached. In both cases, the seeds were all infected, the same as the previous week. The decrease that ocurred between sampling periods in some instances can be explained on the basis of 1) seasonal fluctuations in temperature and moisture; 2) random sampling of pods from different locations on a given plant; and 3) external and internal cracking of pod walls. The cracking permitted mercuric chloride to be absorbed into the pods, resulting in the killing of seed-borne mycelia and spores.

The kind of fungi isolated from soybean seed within the pods was essentially the same in all 4 years (Table 3). The purple-seed-stain fungus, *Cercospora kikuchii*, averaged 50 per cent of the total isolates for the 4

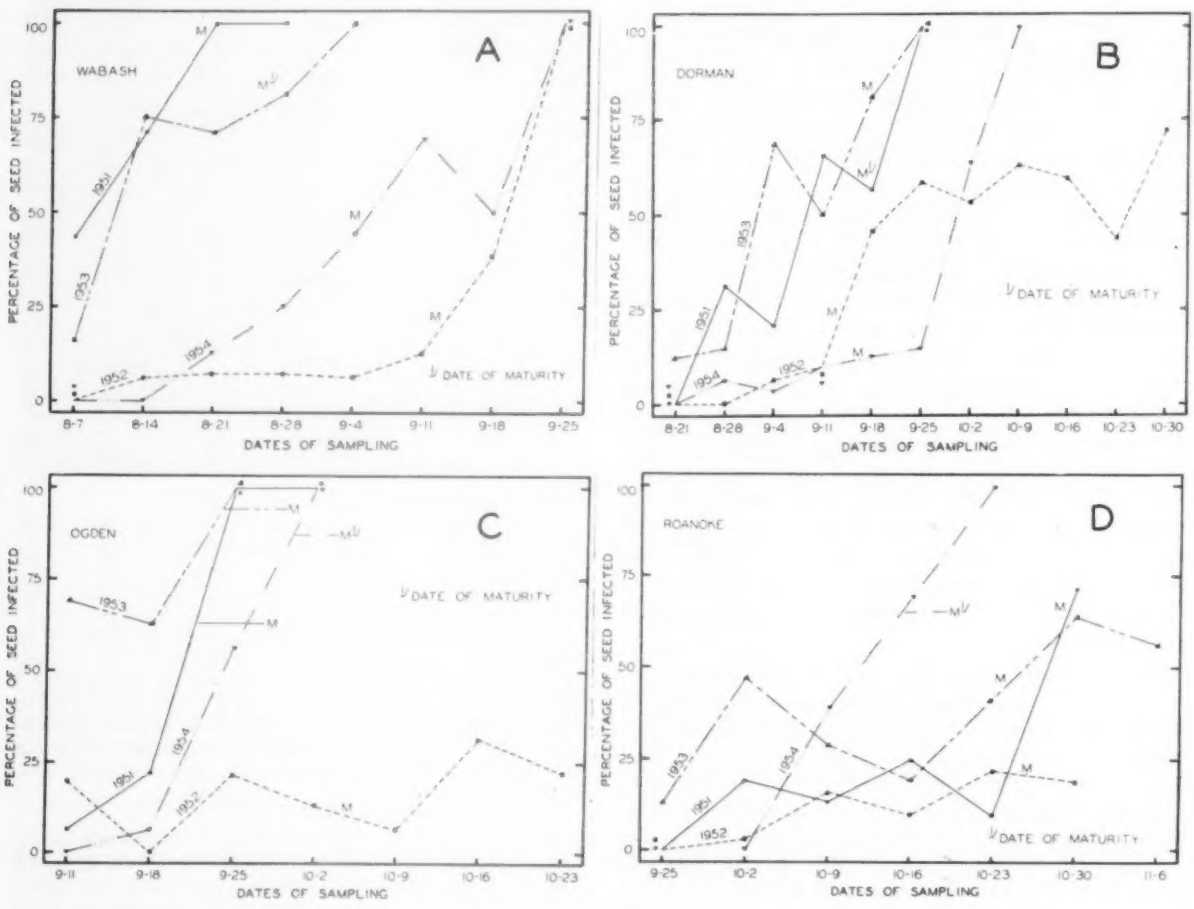

FIG. 3. Summary of results with 4 varieties of soybeans, showing the percentage of seed infected with fungi while seed were still within the pods, Stoneville, Mississippi, 1951-54.

TABLE 3.—*Fungi isolated from seed within the pods of different varieties and strains of soybeans and their relative prevalence expressed as percentage of the total isolates, Stoneville, Mississippi, 1951-54*[a]

Fungus	Percentage of total isolates			
	1951	1952	1953	1954
Cercospora kikuchii	55	35	64	40
Fusarium oxysporum	20	10	9	10
Alternaria spp.	12	36	14	39
Phomopsis sojae	8	1	3	6
Cladosporium spp.	?[b]	13	6	2
Miscellaneous species[c]	2	4	4	2
Unidentified species	3	1	Tr	1

[a] Percentages represent an average of 17, 15, 13, and 13 surveys made at weekly intervals and involving 10, 12, 18, and 10 varieties and strains of soybeans, respectively, in 1951-54. Of 11,800 seeds from within pods plated out over the 4 years, fungus isolates were obtained from 5200 or 44.1 per cent.

[b] Included with unidentified species.

[c] *Phyllosticta* spp., *Aspergillus niger*, *Aspergillus* spp., *Fusarium roseum*, *F. solani*, *Curvularia lunata*, *C. affinis* (?), *Chaetomium* sp., *Nigrospora* sp., *Penicillium notatum* Westling, *Penicillium* spp., *Rhizopus nigricans*, *Trichoderma* sp., *Sclerotium bataticola* Taub., *Helminthosporium* spp., and *Colletotrichum destructivum* were isolated from seed during 1 or all of the 4 years but none exceeded 1 per cent of the total isolates.

years, although the purple discoloration was observed only infrequently in the field. Other fungi were less prevalent and varied from season to season. *C. kikuchii* was more commonly isolated from later maturing varieties and strains than from early maturing ones. Ogden yielded a higher percentage of isolates of *C. kikuchii* than did any other variety sampled. In general, Ogden showed a greater percentage of purple-stained seeds than did other varieties, especially Roanoke and Lee, which showed only a few discolored seeds. Seventy-seven per cent of the isolates from CNS (which has not shown visible purple discoloration in the field) in 1951 were *C. kikuchii*. When seeds of CNS and Ogden were plated on agar, the purple discoloration developed within 5 days. In comparison to Ogden, which is very susceptible to purple stain, CNS showed resistance when inoculated in the laboratory. Apparently this variety contains some substance that prevents development of the purple stain in the field but only inhibits it under laboratory conditions. *Phomopsis sojae* was most frequently isolated from early maturing varieties and strains. *Fusarium oxysporum* and *Alternaria* spp. were frequently isolated from seeds removed from insect-injured pods or from pods that showed a considerable amount of wall cracking, both external and internal.

The distribution of fungus infection among seeds at various positions within the pods was studied each year. *C. kikuchii*, *Alternaria* spp., and *F. oxysporum* were isolated in approximately equal percentages from the 3 locations within the pods. *P. sojae* was isolated most frequently from the seed nearest the peduncle and least frequently from the seed nearest the tip of the pod. This suggests a systemic type of penetration. *C. kikuchii* was isolated from immature seeds not showing the visible purple discoloration and also prior to development of external pod-spotting symptoms. This is suggestive of a delayed type of systemic infection, similar to that described for species of *Colletotrichum* by Tiffany (9). *Alternaria* spp. and *F. oxysporum* were frequently isolated from insect-punctured seeds.

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PATHOGENIC VARIABILITY WITHIN CLEISTOTHECIA OF ERYSIPHE GRAMINIS[1]

H. R. Powers, Jr., and J. G. Moseman[2]

SUMMARY

Variation in pathogenicity within *Erysiphe graminis* f. sp. *tritici* and *E. graminis* f. sp. *hordei* was determined by the reactions of differential varieties of wheat and barley to 106 cultures derived from single ascospores. A wide range in pathogenicity was observed. As many as 3 pathogenic types were derived from a single cleistothecium. Fourteen distinct pathogenic types were identified among 53 cultures of *E. graminis* f. sp. *hordei*, whereas 7 were obtained from 56 cultures of *E. graminis* f. sp. *tritici*.

Powdery mildews of wheat and barley, caused by *Erysiphe graminis* DC. f. sp. *tritici* Em. Marchal and *Erysiphe graminis* DC. f. sp. *hordei* Em. Marchal, respectively, have become more prevalent and destructive in the eastern half of the United States during the last decade. This has been attributed primarily to the spread of new, or previously rare, races of the mildew organisms (13). Varieties such as Hardired wheat and Sunrise barley, which at the time of their release were resistant to the prevalent races of the pathogens, have been heavily attacked by new races that have appeared since then. In view of this situation it was obvious that information regarding the genetics of the pathogen, as well as of the host, would be an aid in producing varieties with lasting resistance.

The aspect of the problem regarding resistance in the host has been investigated by many workers (9, 10, 11); however, the critical factor of the pathogenic variability of this organism has not been previously examined, except for the work on the identification of races. These experiments were designed to investigate the range in pathogenicity of *E. graminis* among cultures from single ascospores within individual cleistothecia.

Numerous workers have reported the existence and prevalence of pathogenic races of *Erysiphe graminis*. Mains and Dietz (4) first demonstrated the presence of physiologic races of *E. graminis* f. sp. *hordei*. Investigations regarding the races of this organism have been reviewed by Moseman (5). Waterhouse (15) first noted the existence of races within *E. graminis* f. sp. *tritici*. Mains (3) subsequently demonstrated the presence of 2 races of the wheat mildew fungus in the United States. Schlichting (12) in Germany, Vallega (14) in Argentina, and Newton and Cherewick (7) in Canada also reported on races of *E. graminis* f. sp. *tritici*. Later investigations in this country were made by Taylor et al (13) and Lowther (2).

The variability obtained when ascospores are used as inoculum has not been studied very extensively. Cherewick (1), however, reported that 4 races of the barley mildew fungus were obtained from ascospores of this organism, whereas only 2 races were found in conidial collections made in the same area.

METHODS.—Cleistothecia were collected in the field at several locations. Individual fruiting bodies were matured and then suspended over water agar. When ascospores were discharged, individual spores were picked from the agar surface and transferred to leaves of susceptible varieties (Little Club wheat, C. I. 4066, and Heil's Hanna barley, C. I. 682).[3] The details of maturation and inoculation techniques have been given by Moseman and Powers (6). Ten per cent or less of the ascospores transferred were successful in establishing infections. In all, 53 cultures from single ascospores of *E. graminis* f. sp. *tritici* were obtained from a total of 18 cleistothecia, and 53 cultures of *E. graminis* f. sp. *hordei* from 15 cleistothecia.

The cultures were increased on susceptible plants and then used to inoculate the differential varieties employed for the identification of races of the mildew fungi. For the wheat pathogen, these varieties are Axminster, C. I. 8195; Chul, C. I. 2227; Hope, C. I. 8178; Normandie, C. I. 12747; and Ulka, C. I. 11478. The varieties Huron, C. I. 12663, and Norka, C. I. 4377, also were included in the test as they had been used as differentials by other workers. The data from these 2 varieties are not presented, however, since Huron gave a susceptible reaction to all cultures, and the reaction of Norka was identical with that of Axminster. The 6 standard differentials for barley mildew are Black Hulless, C. I. 666; Chevron, C. I. 1111; Goldfoil, C. I. 928; Heil's Hanna, C. I. 682; Nepal, C. I. 595; and Peruvian C. I. 935.

Of these 6 varieties, only 4 gave differential readings. Heil's Hanna was uniformly susceptible to all cultures, whereas Goldfoil was uniformly resistant. Data from these 2 varieties are omitted. In addition to the barley varieties listed above, the 3 varieties Hanna, C. I. 906; Kwan, C. I. 1016; and Duplex, C. I. 2433, were included in the tests. Since the pathogenic variation discussed in this paper was detected by use of both the standard differentials and 3 additional varieties, the term "pathogenic type" will be substituted for "race."

During inoculation and incubation, each culture was

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[3] C. I. refers to accession numbers of Cereal Crops Section, Field Crops Research Branch.

TABLE 1.—*Reactions of wheat differential varieties to 3 cultures derived from a single cleistothecium of Erysiphe graminis f. sp. tritici*

Differential variety	Reaction[a] of variety to indicated culture		
	A–1	A–2	A–3
Axminster	3–4	0	3–4
Chul	0	0	0
Hope	3–4	3	3
Normandie	3	0	0
Ulka	4	3–4	0

[a] 0 = Immune, 1 = highly resistant, 2 = moderately resistant, 3 = moderately susceptible, and 4 = very susceptible.

kept isolated within a 2x2x2-ft. cage constructed of plastic material known commercially as "Wyroglass." The tops of the cages were covered with 4 layers of cheesecloth. These cages were located in a greenhouse where the temperatures were held at approximately 70°F. Readings[4] were made 7–10 days after inoculation. Two separate tests were made with each single-ascospore culture. In every case the 2 inoculations gave nearly identical readings.

RESULTS.—Data obtained from the inoculation of differential varieties with the 106 cultures of *E. graminis* revealed a wide range of pathogenic variability. This variation existed among cultures derived from an individual cleistothecium, as well as among cultures obtained from different cleistothecia. The heterogeneity of cultures derived from a single cleistothecium was particularly evident in the case of *E. graminis* f. sp. *tritici*. Two or more pathogenic types were found in every test in which at least 3 cultures were obtained from a single fruiting body. In many cases each of the 3 cultures obtained from a single cleistothecium gave a different reaction on the differential varieties, an example of which is shown in Table 1.

Variability also was common in *E. graminis* f. sp. *hordei*. Several cleistothecia produced cultures of as many as 3 pathogenic types. Eight distinct pathogenic types were observed among the 19 cultures obtained from cleistothecia collected in New York and Pennsylvania. One of these, from Ithaca, New York, has not been previously reported and has been designated race 20. The reactions of the differential varieties to these cultures, as well as to those obtained from cleistothecia collected in the southeastern states, are presented in Table 2. Among the 53 cultures tested 14 pathogenic types were observed, as indicated by the reactions given by 4 of the standard differential barley varieties and the 3 additional varieties.

The reactions of the wheat differential varieties to cultures of *E. graminis* f. sp. *tritici* obtained from cleistothecia collected at Beltsville, Maryland; Kearneysville, West Virginia; and Warsaw, Virginia are presented in Table 3. Seven pathogenic types were found among 56 cultures; however, all cleistothecia were collected within 125 miles of Beltsville, Maryland, so that a relatively small area was sampled.

DISCUSSION.—A wide range of pathogenic variability within *Erysiphe graminis* was indicated by the results obtained in practically all of the tests. The least pathogenically diverse culture of wheat mildew used in the experiments produced a susceptible reaction on only one of the differential varieties, whereas the most widely pathogenic culture caused susceptible reactions

[4] A detailed description of barley mildew readings has been published by Moseman (5). The general description of reaction types for both wheat and barley mildew is as follows: 0 = immune, 1 = highly resistant, 2 = moderately resistant, 3 = moderately susceptible, and 4 = very susceptible.

TABLE 2.—*Reactions of 7 barley varieties to cultures of Erysiphe graminis f. sp. hordei collected in southeastern and northeastern United States*

Reactions[a] on indicated variety							Number of cultures (from 2 areas) of each pathogenic type	
Black Hulless	Chevron	Nepal	Peruvian	Hanna	Kwan	Duplex	Southeast[b]	Northeast[c]
1–2	4	1–2	0–1	0	4	4	9	0
1–2	4	1–2	0–1	0	2	4	9	0
1–2	4	1–2	0–1	4	2	4	8	0
1–2	4	1–2	0–1	0	2	2	5	0
1–2	4	1–2	0–1	0	4	2	1	0
0–2	0–1	0–1	0–1	0	2	0	1	0
3	3–4	3–4	0–1	0	2	4	1	1
3	3–4	3–4	0–1	4	2	2	0	9
3	3–4	3–4	0–1	4	2	0	0	3
1–2	4	1–2	0–1	4	2	2	0	2
3	3–4	3–4	0–1	0	2	0	0	1
3	3–4	3–4	0–1	4	2	4	0	1
3–4	4	2	2	4	2	2	0	1
4	4	2	4	4	2	2	0	1[d]

[a] 0 = Immune, 1 = highly resistant, 2 = moderately resistant, 3 = moderately susceptible, and 4 = very susceptible.
[b] North Carolina, Virginia, Mississippi, and Tennessee.
[c] New York and Pennsylvania.
[d] Previously unreported race 20 (Reaction on Goldfoil, type 0; on Heil Hanna, type 4).

TABLE 3.—*Reactions of the 5 differential wheat varieties to cultures of Erysiphe graminis f. sp. tritici collected within 125 miles of Beltsville, Maryland*

Reaction[a] on indicated variety					Number of cultures (from 3 areas) of each pathogenic type		
Axminster	Chul	Hope	Normandie	Ulka	Md.[b]	W.Va.[c]	Va.[d]
0	0	3–4	0	3–4	11	15	3
0	0	3	0	0	4	7	4
3–4	0	3	0	0	3	0	0
0	0	1–2	0	1–2	2	0	0
3–4	0	3–4	3	4	2	0	0
3–4	4	4	3	4	2	0	0
0	0	0	0	4	0	0	3

[a] 0 = Immune, 1 = highly resistant, 2 = moderately resistant, 3 = moderately susceptible, and 4 = very susceptible.
[b] Beltsville, Maryland.
[c] Kearneysville, West Virginia.
[d] Warsaw, Virginia

on all of the differentials. The cultures of the barley mildew fungus derived from cleistothecia collected in the Northeast also proved to be extremely variable, yet the number of cultures involved in this case represented a very small sample of the mildew pathogen population in that region. The fact that a high percentage of these cultures gave different pathogenic reactions indicates a highly heterogeneous population.

Perhaps even more striking was the variability observed within groups of cultures derived from a single cleistothecium. This was particularly evident since relatively small numbers of single-spore cultures were available for use in detecting variability within any one fruiting body. Usually, less than 100 spores were discharged from a single cleistothecium, and of these, 10 per cent or less were successful in establishing infections. In the case of the data presented in Table 1, the 3 cultures were obtained from a total of 80 discharged ascospores. Since each of these 3 cultures incited different host reactions, the total variability within the population of 80 ascospores could conceivably have been much greater than that observed. Many other cleistothecia, of both the wheat and barley pathogens, were found to produce cultures of as many as 3 pathogenic types.

Erysiphe graminis is known to be heterothallic (8), therefore, the conjugation of 2 compatible cultures is necessary for the completion of its life cycle. Hybridization of strains with different pathogenic potentials would be probable under field conditions. The genes controlling pathogenicity have been found to operate independently of those governing compatibility (8). This would permit the combination of a wide range of pathogenic types. Much of the variability observed in the current tests, therefore, probably can be attributed to hybridization with subsequent recombination and segregation.

Under these conditions, new races of the mildew fungi may be expected to appear continually as threats to commercially grown varieties. A thorough study of the genetic basis of pathogenicity in the organism, as well as resistance in the host, may offer a practical approach to the problem of combating powdery mildew. Information obtained from such studies would simplify the development of new varieties with combined resistance to a greater range of pathogenic types of the mildew organism. Also, shifts in the population of pathogenic types in any given region, as new varieties are released, might be predicted with greater certainty.

PLANT INDUSTRY STATION
BELTSVILLE, MARYLAND

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EFFECTS OF INSECTICIDES AND PHYSICAL BARRIERS ON FIELD SPREAD OF PEPPER VEINBANDING MOSAIC VIRUS[1]

John N. Simons[2]

SUMMARY

Field plot trials, in which nightshade (*Solanum gracile* Link) was used as a primary source of veinbanding mosaic virus, pepper as a test plant, and naturally occurring aphids as vectors, were conducted during the fall and spring of 1954-55. Evidence showed that a distance of 150 ft. between plots could, under the conditions present, effectively limit the spread of veinbanding mosaic virus. The use of parathion or demeton in the plots failed to give control.

When parathion was used as an aphicide on the primary source of virus (nightshade), virus spread was reduced significantly. In these tests, potted healthy peppers were taken to the field for short periods of exposure to viruliferous aphids after which time they were returned to the greenhouse for completion of the incubation period.

The effect of placing a vertical physical barrier between the source of inoculum and the crop plant was investigated to a limited extent. Use of sunflowers as barrier plants resulted in a significant decrease in virus spread. Demeton sprayed at 3-week intervals on sunflowers bordering pepper plots did not significantly reduce virus spread in peppers over that obtained in plots bordered with unsprayed sunflowers. A statistically significant correlation was found between row position and the amount of infection in the sunflower-protected areas. Spread to peppers next to sunflowers was about half as fast as spread to peppers in the center of the plots.

During the past 2 decades, much valuable information has been revealed regarding the relationships of aphid vectors to the transmission of nonpersistent viruses. Although the actual mechanics of transmission are still incompletely understood, it would seem that a good deal of the pertinent vector-virus information is available insofar as field control is concerned. The most obvious weak link in the cycle of transmission, from the standpoint of the vector, appears to be the relatively short time the insect remains infective after having acquired virus. Because of this, the experiments described herein were conducted to determine, if possible, the effects of such phenomena as distance from virus source, effects of insecticides on natural sources and crop plants, and the effect of a barrier crop on virus spread. In order to simulate natural spread of the virus, all the work was done under field-plot conditions, with a known native weed host (nightshade) as the virus source.

MATERIALS AND METHODS.—The virus used was the pepper veinbanding mosaic virus described earlier (5) and characterized as belonging to the nonpersistent aphid-borne group. Nightshade (*Solanum gracile* Link), a known native weed source (5), was mechanically inoculated and used as the primary source of inoculum in all cases. Pepper (*Capsicum frutescens* L. var. California Wonder) was used as the test species. Cultural practices used in growing the peppers were similar to those recommended by the Everglades Experiment Station for commercial production.

Disease ratings were made at weekly intervals in nearly all cases, and plants were charted individually so that an accurate record of the pattern of spread could be obtained.

RESULTS.—*Effect of insecticides applied to the crop plant on spread of virus.*—Several workers have reported on the use of aphicides as a means of controlling nonpersistent aphid-borne viruses (3,4,6,7). Although economic control of henbane mosaic through the use of nicotine was reported by Watson (7), usually no appreciable reduction in spread of nonpersistent viruses has resulted from the use of aphicides. In order to obtain information on the control of a virus of this type under south Florida conditions, the following experiment was carried out.

An open area (no trees nor buildings within ½ mile) with the approximate dimensions of 550 × 840 ft. was laid out into six 50 × 80-ft. blocks, each separated on all sides by 150 ft. of clean-cultivated ground (Fig. 1,A). Three blocks were bordered centrally with a row of diseased nightshade plants (8 plants spaced about 12 ft. apart) and the other 3 had no nightshade next to them. Each block consisted of 9 plots, and each plot contained two 20-ft. rows of transplanted peppers (14 plants per row). The plots were incorporated into a 3 × 3 Latin square and were composed of 1) 3 plots sprayed at 2-week intervals with 23.0 per cent demeton concentrate (0,0-diethyl-(2-ethylmercaptoethyl) thiophosphate) at the rate of 2 pints/100 gal. of water, 2) 3 plots sprayed at 2-week intervals with 25 per cent parathion emulsion concentrate 0,0-diethyl-0-*p*-nitrophenyl thiophosphate) at a rate of 1.5 pints/100 gal. of water, and 3) 3 unsprayed plots. The insecticides were applied with the small-

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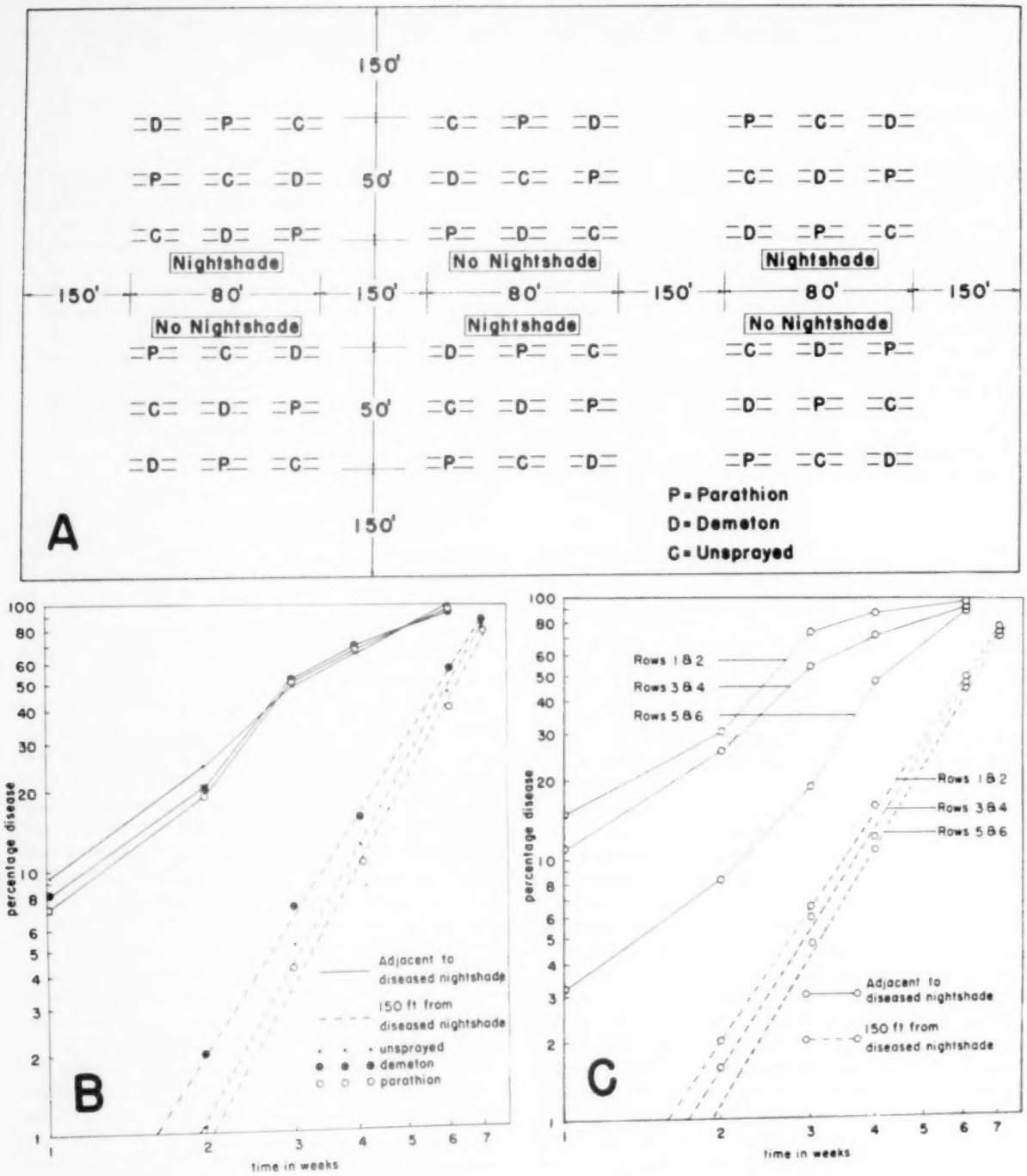

FIG. 1. A) Plot diagram for experiment designed to determine effect of spraying insecticides on peppers and effect of distance from nightshade source of virus on spread of pepper veinbanding mosaic virus. B) Percentage of plants diseased (at intervals during the season) in sprayed and unsprayed rows adjacent to and 150 ft. from infected nightshade. C) Percentage of plants diseased (at intervals during the season) in rows located at various distances from infected nightshade.

plot spray rig described by Cox et al (1); the coverage obtained was excellent.

After the first infections were found on November 9, the pepper plants were examined individually at weekly intervals (except for the week of Dec. 6). Aphids were counted on the peppers at 2-week inter-

vals. Counts were made on the basis of 3 leaves (upper, middle, and lower) on each of 10 plants in each of the 9 plots. At no time did the population approach a level of economic importance. The highest population was observed on November 8, when the counts for the check plants averaged 1.85 aphids per leaf. Aphid control in the sprayed areas was nearly 100 per cent effective, with only alate individuals observed in most instances. The principal species present was the cotton aphid, *Aphis gossypii* Glover.

No discernible decrease in virus spread resulted from the use of aphicides (Fig. 1,B). In view of the very low population of aphids in the unsprayed treatments, this is not too surprising. Where the efficiency of transmission is as high in short feeding periods as is apparently the case with this virus, a very low population of winged aphids might be expected to cause rapid spread in spite of the presence of a powerful aphicide.

Effect of insecticide applied to the weed source on spread of virus.—The above results indicate that little would be gained through spraying the crop or test plant with an insecticide insofar as control of veinbanding mosaic virus is concerned. Another approach to the problem, however, might be to spray the weed host plant with the aphicide. A field situation, which suggested some merit to this line of attack, was noted during the months of October and December, 1954. Dr. R. S. Cox had maintained a block of peppers on a commercial farm near Canal Point, Florida, for testing antibiotics. His plots were sprayed once a week with parathion, and aphid control was excellent. Nevertheless, veinbanding mosaic virus spread at a rapid rate through the plots. The primary source of virus appeared to be infected plants that were inadvertently transplanted to the center of the plots. Along 1 side of the plots, a drainage ditch separated the area from a neighboring pepper field. Although only 60–70 ft. separated the 2 plantings, for some reason the virus failed to move beyond the edge of the sprayed plots. Approximately ½ the plants in the plots were infected with virus by mid-December, but still there was no disease in the adjacent area[3]. One possible explanation of this situation was that the parathion applications had affected the aphids such that they could no longer fly in a normal manner. Within-field spread in the plot area might not be noticeably reduced, but the effect on a neighboring field could be more evident. In order to test this hypothesis, the following experiment was carried out.

Two 40-ft. rows of mechanically inoculated nightshade plants (22 plants per row) were set out 150 ft. apart (Fig. 2). They were located in a clean-cultivated area with dimensions of approximately 500 × 700 ft. These plants were allowed to grow until they formed 2 solid hedges. Pepper seedlings, which were grown in 4-in. clay pots to the 6- to 8-leaf stage in the greenhouse then set outside for several days in order to harden off, were placed (in the pots) in the field adjacent to the diseased nightshade. They were placed in 12 rows alongside each of the nightshade hedges (Fig. 2); the rows were 4 ft. apart and 40 ft. long, with each consisting of 21 pepper plants. These plants were left in the field for 6 days before being brought back to the greenhouse to complete the incubation period. Because the incubation period of the disease in pepper is normally 12–14 days, removing the plants from proximity to the virus sources after 6 days minimized the complication of secondary spread (pepper to pepper). While the plants were in the field, one of

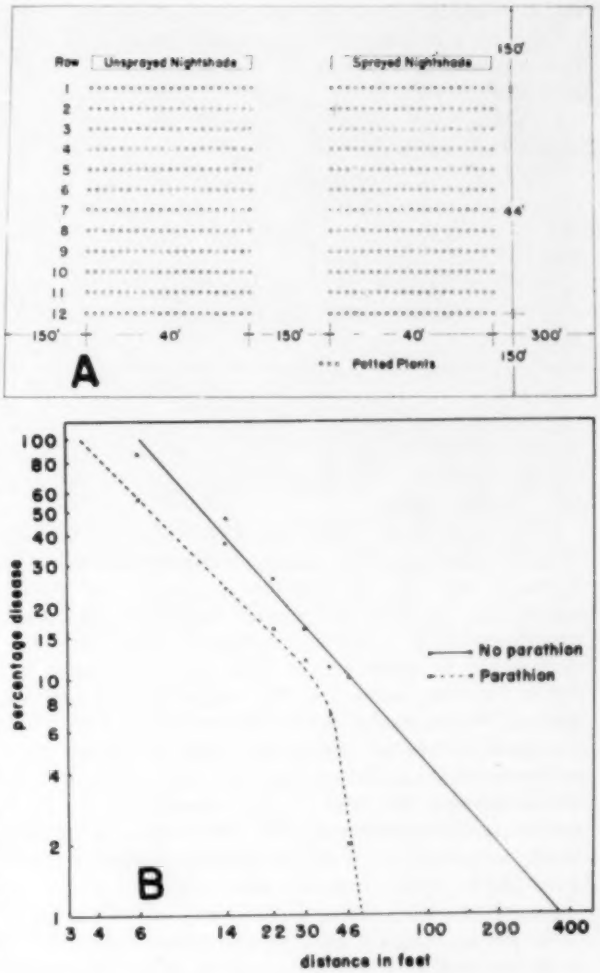

FIG. 2. A) Plot diagram of experiment designed for the evaluation of the effect on virus spread of parathion when applied to the weed source. B) Results showing decreases in total amount of disease and in distance the virus was carried in the parathion-sprayed plots.

[3] These plots were originally laid out to test the effectiveness of streptomycin sprays against bacterial leaf spot of pepper. Although some treatments consisted of weekly applications of 500- and 1000-ppm material, no indication of virus control was evident in any treatment. The author is indebted to Dr. R. S. Cox for use of these data.

the nightshade hedges was sprayed twice (3-day intervals) with a 25 per cent parathion emulsion used at a concentration of 1.5 pints per 100 gal. of water. Both nightshade hedges remained free of aphid colonies during the course of the experiment. The results are shown in Figure 2.

There was a considerable decrease in total transmission where the parathion was used (170 infected of 941 set out versus 269 of 972, $\chi^2 = 14.2$, $P = <0.01$). From this it seems that the insecticide affected the aphids such that many were unable to fly from the diseased nightshade to the peppers. Presumably, the fact that the aphids were affected by the insecticide while in the process of acquiring virus could have considerable bearing on explaining the differences between the number of transmissions that were obtained in this experiment and that obtained in the preceding one in which the test plants (peppers) were sprayed with an insecticide.

Effect of distance from source of inoculum on spread of virus.—In both of the above experiments, certain information was obtained on the distances viruliferous aphids can fly and still remain infective. The first experiment (Fig. 1, A) was designed so that observations could be made on the effect that a distance of 150 ft. between source and host might have on rate of spread. These results are shown in Figure 1, C. Although virus spread seemed to follow the same general-type curve ($y = cx^m$), there was a lag of 2–4 weeks between the time a given amount of infection was present in the plots adjacent to the diseased nightshade and the time it was present in the plots that were 150 ft. distant from the virus source.

During the sixth week, a differential of 98 per cent to 48 per cent existed between the 2 treatments (LSD = 13 per cent, $P = 0.01$). Disease incidence increased very rapidly in the distant areas after this date, presumably because of the many diseased pepper plants in the plots. Thus, secondary spread (pepper to pepper) became increasingly more important, eventually overshadowing the pattern of primary spread (nightshade to pepper).

It should also be noted that the incidence of disease in the plots was closely correlated with the proximity of the plants to the virus source. In the plots adjacent to the nightshade, there was a well-defined gradient of disease incidence away from the source; in the plots 150 ft. away, this pattern of spread was still somewhat evident (Fig. 1, C).

Additional information on the effect of distance from source on spread was obtained from the experiment in which the inoculum plants were sprayed with parathion (Fig. 2). Because the data obtained here were not obscured by secondary spread, it is believed that some estimate of the distance a winged aphid might be expected to travel under the prevailing conditions before becoming noninfective can be calculated. In the case of the aphids that fed on the nightshade not sprayed with parathion, there still was considerable infection present at the outer limit of the plot. Extrapolation of the regression line indicates that some aphids could retain infective virus after moving as far as 400 ft. from the source. That this regression line dropped from 90 per cent at 6 ft. from the virus source to 10 per cent at 50 ft. might also indicate that the aphids carried the virus for a distance of no more than several hundred feet. Where the insects fed on parathion-sprayed source plants, this movement seemed sharply curtailed at 50 ft. This difference suggests that the insecticide had some adverse effect on the aphids' ability to fly.

A marked difference on the effect of distance on virus spread between the fall and spring experiments is indicated. Whereas 150 ft. of distance almost checked primary virus spread under the fall conditions, this same distance only reduced primary spread to a level of 3–4 per cent in 1 week under the spring conditions (Fig. 2). It is believed that at least 2, possibly 3, factors accounted for this. First, there definitely were many more aphids in the spring than in the fall. This would tend to extend the distance slightly; however, it is doubtful that increased population alone would lead to a doubling of the radial distance. Second, the air temperature was higher in the spring than in the fall. The mean air temperature from November 9 to December 20 was approximately 62.3°F; from April 11 to May 16, it was approximately 72.7°F. It may be that the aphids flew greater distances when the temperature was higher. Davies (2) has reported that temperatures of less than 64°F greatly restrict numbers of aphid flights of *Myzus persicae* (Sulz.) in the laboratory.

It should also be pointed out that the clean-cultivated area around the plots could have affected the distance that aphids carried the virus. If the plots had been surrounded by a grass or other nonsusceptible plant cover, there could have been a reduction in the distance the insects moved before alighting. A more plausible explanation of the effect of the clean-cultivated area on transmission is that the winged aphids became starved while passing over it and thus their efficiency as potential vectors was increased.

Effect of a physical barrier on spread of veinbanding mosaic virus.—Since nonpersistent aphid-borne viruses are, in general, retained for periods of less than an hour by their vectors, an efficient means of deterring aphids from landing on susceptible plants for a short time after acquisition of virus might result in decreased transmission. Under laboratory conditions, veinbanding mosaic virus is lost in most cases after a 30- to 60-minute period of starvation subsequent to acquisition feeding (5). Sunflower (*Helianthus annuus* L.) is nonsusceptible to the virus and is commonly grown as a windbreak in this area, hence small plots of pepper were surrounded by sunflower hedges in order to determine what effect, if any, this might have on aphid movement and thus on transmission of veinbanding mosaic virus.

A clean-cultivated area, approximately 550 × 850 ft., and consisting of 6 blocks, separated by 150 ft. of

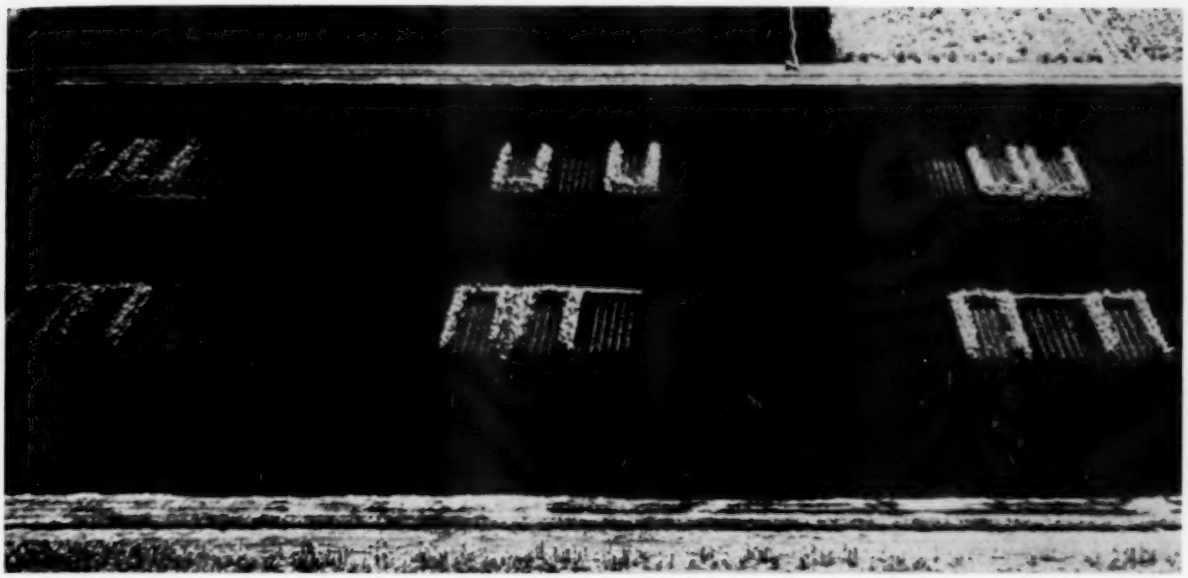

FIG. 3. Aerial photograph showing experimental design employed in obtaining information on effect of sunflower barriers on spread of veinbanding mosaic virus. Note rows of diseased nightshade in front of blocks at upper left, lower center, and lower right.

clean-fallowed land on all sides was laid out (Fig. 3). A block consisted of 3 plots, each of which contained five 60-ft. rows of field-seeded pepper spaced 4 ft. apart. The plants were spaced about 1 ft. apart in the rows so that a total of 300 was used in each plot. Plots were separated by 10-ft. alleyways and treated as follows: 1) bordered on 3 sides with sunflowers that were sprayed at 3-week intervals with a 23 per cent demeton concentrate at the rate of 2 pints/100 gal. of water, 2) bordered similarly with sunflowers that were not sprayed with insecticide, and 3) not bordered with sunflowers. In 3 of the 6 blocks, 12 mechanically inoculated nightshade source plants were located along the central border (Fig. 3). Disease counts were taken at weekly intervals beginning on April 11 and continued until May 16.

During the fifth week, 95 per cent of the plants in the unprotected check plots were infected, whereas 63 per cent of those in plots bordered by unsprayed sunflowers were infected (Fig. 4, A). The presence of the sunflowers significantly reduced the spread of virus (LSD = 23 per cent, P = 0.01). In general, about 10 more days were required for disease incidence to reach a given level in the sunflower-protected area than were required in the check plots. Eventually, all of the plants became diseased.

The demeton applications resulted in slightly less disease than was present in the plots bordered with unsprayed sunflowers, but the decrease was not statistically significant.

The principal manner in which the sunflowers caused the reduction of virus spread was apparently as a mechanical barrier to the aphids. This was particularly noticeable in the rows adjacent to the barrier. In Fig. 4, B, the distribution of disease by row in the plots is shown for the week of May 9, the first week in which approximately 100 per cent of the plants in the check plots were recorded as diseased. Considerably less disease appeared in the outer rows (next to sunflowers) than in the 3 central rows. The outer rows are almost invisible from the air (Fig. 3) because of the spreading habit of growth of the sunflowers. Evidently the sunflowers afforded sufficient protection to these rows so that aphids alighted in them less frequently than in the center rows. This also indicates that more aphids flew over the barrier than through it, since a more equal distribution of disease might be expected if most of the insects went through the barrier. The fact that the same phenomenon occurred to a similar degree regardless of the use of demeton or to proximity to the nightshade source would further indicate that the sunflowers were mainly responsible for the results obtained.

DISCUSSION.—Two types of field spread are involved in the transmission of a nonpersistent aphid-borne virus. These are often called 1) primary spread (from outside the field to within the field) and 2) secondary spread (within-field). In general, the results reported here have been concerned with phenomena associated with primary spread of virus.

Among the several factors that seem most important in causing primary spread are 1) numbers of alate vectors present, 2) vector efficiencies of the aphid species, 3) relative amount of movement of the aphids (both in terms of total flights per unit of time and the distance flown in each flight), 4) the number of diseased plants present (both in terms of density per unit area and proximity to the susceptible crop plants, and

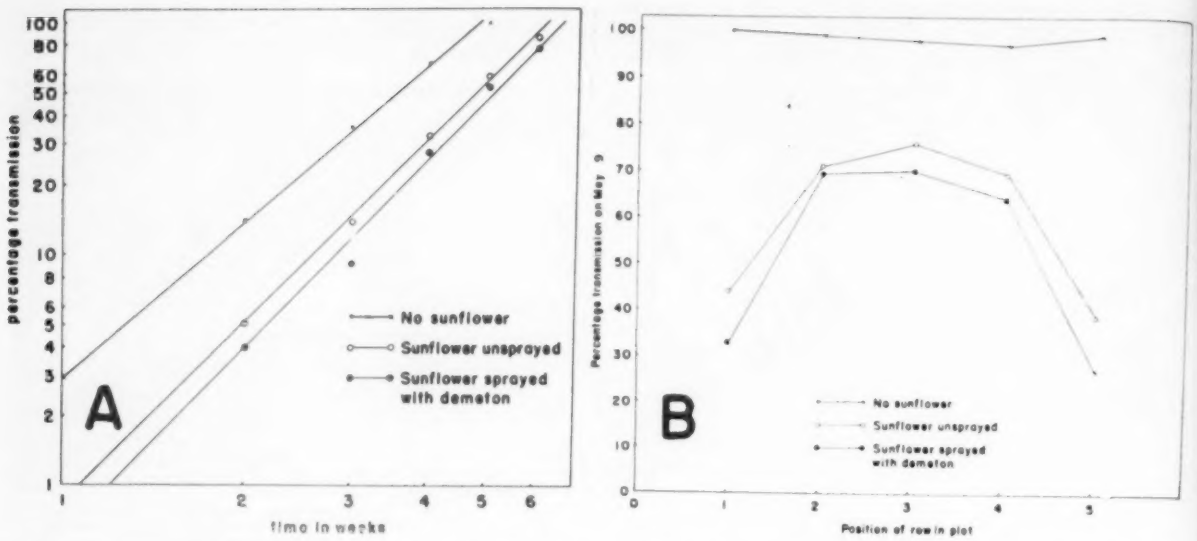

FIG. 4. Percentage transmission of veinbanding mosaic virus to peppers bordered by a barrier of demeton-sprayed sunflower plants, to peppers bordered by unsprayed sunflowers, and to peppers not bordered by sunflowers. A) Disease incidence at weekly intervals beginning April 11. B) Disease incidence according to position of rows in the plot. In plots bordered by sunflowers, rows 1 and 5 were the ones adjacent to the sunflowers.

5) climatological factors. Undoubtedly many other factors, such as the relative potency of the virus source species, the attractiveness of the plant species to the aphid, and the topography of the area, also influence virus spread. It seems logical that quantitative changes in any of these factors could result in varying amounts and rates of virus spread.

Observations that have been made in the field during the last 3 years on spread of veinbanding mosaic virus have given indications of the importance of several of these. It now seems apparent that primary spread is a much more serious problem in the spring than in the fall and winter. This observation has been made before in other areas and has been generally attributed to the presence of more aphids and more diseased plants in the area in the spring than are present in the fall and winter. Undoubtedly, increases in the levels of these 2 factors are responsible for some of the increased transmission observed in the warmer time of year; however, another equally, if not more important, quantity also seems to be involved. This is the influence of temperature on the distance that the aphids fly, and thus on the distance that virus is carried.

During the spring of 1956 (average temperature during this period was 72.3°F), a field situation that could be interpreted to demonstrate this phenomenon rather clearly was noted. An area of about 75 × 275 ft. had been planted with peppers arranged in 4 blocks (north to south) of 13 50-ft rows. Twenty-five-ft. alleys separated the blocks. On May 9, 70 per cent of the plants in the north block were infected, 63 per cent of those in the next block, 57 per cent of those in the third block, and 45 per cent of those in the fourth (south) block. The variation in amount of transmission on a row-to-row basis (east-to-west direction) was negligible. This pattern of spread seemed to indicate that the virus source was north of the plots and that the virus source was a good distance away (because of the gradual decrease in disease incidence from plot to plot). The latter is thought to be true also because all nightshade had been rogued for a distance of 700 ft. north of the plots. Readings were taken 1 week later on May 16, and the counts were as follows: north plot, 100 per cent; next plot, 98 per cent; third plot, 98 per cent; and fourth (south) plot, 86 per cent. It is apparent that spread had been very rapid during this 1-week period.

A very large number of cotton and green peach aphid alates were present during this time. Therefore, one is led to suspect that this extensive spread of the disease was either the result of a great number of infective individuals landing in the field, or that a few infective aphids had brought in the virus and that the bulk of the spread was of a within-field type caused by large numbers of winged aphids feeding on these relatively few diseased pepper plants. The latter explanation seems likely, for it is supported by the observed gradient from plot to plot.

From a practical standpoint, the observation that an aphid can bring virus in from a distance of nearly 800–1000 ft. during warm weather as compared to only 150–200 ft. during cold weather could have important implications. This increased activity, together with the larger numbers of aphids usually present during the warmer season, could result in failure of a weed-control program that might be successful during a cool time of year.

Certainly the evidence indicates that at this time any control program for veinbanding mosaic virus must be based on eliminating the weed source of virus before the crop season starts. It would seem likely that the use of tall plant barriers around and within the crop would also aid in reducing spread. Certain situations might be favorable for the use of insecticides on source plants, e.g. where the virus is widespread in one field but not present in a neighboring field. Conceivably, all of these procedures should prove to be additive in effect since each is independent in its action.

The problem of within-field spread continues to present a great challenge as well as a stumbling block to virus control. Until some technique of coping with this problem can be devised, it is doubtful that completely effective control of pepper veinbanding mosaic virus will be achieved.

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THREE STRAINS OF CUCUMBER MOSAIC VIRUS AFFECTING BELL PEPPER IN THE EVERGLADES AREA OF SOUTH FLORIDA[1]

John N. Simons

SUMMARY

At least 3 different strains of cucumber mosaic virus are present in California Wonder pepper (*Capsicum frutescens* L.) in the Everglades area of south Florida. Southern cucumber mosaic virus is the strain most prevalent. A second strain causes necrotic oak-leaf patterns on older pepper foliage and at times induces a reddish or yellowish fruit blistering. The third and least common strain produces a calico-like foliage mottle and causes no apparent fruit symptom.

Immunological studies among the 3 strains indicated close relationships. The physical properties determined for the 3 strains were identical: thermal inactivation point of 65°–70°C and dilution end point between 1:10,000 and 1:20,000.

All 3 strains apparently are transmitted in a nonpersistent manner by the cotton aphid, *Aphis gossypii* Glover, and the green peach aphid, *Myzus persicae* (Sulz.). The degree of aphid transmissibility of the strains differs markedly, however, and it is proposed that this may account in part for their relative prevalences in the field.

The previously reported phenomenon (for southern cucumber mosaic virus) of changing of transmissibility by altering such factors as vector species, inoculum source species, or test species was demonstrated for all 3 strains.

During the course of surveys in south Florida, 4 apparently different mechanically transmissible viruses have been found. One of these appears to be closely related to potato virus Y (6) and threatens the pepper industry. A second virus (5,9), also of considerable economic importance, appears to be closely related to southern cucumber mosaic virus (SCMV). The other 2 viruses, although sporadic in occurrence, are widely distributed in the area. One of them (PCV) causes a calico mottle in pepper and the other (POLV) produces necrotic oak-leaf patterns on the senescent leaves of pepper.

Since the presence of either PCV or POLV is usually correlated with that of SCMV in the field, it

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was thought desirable to work with all 3 viruses in order to determine whether they are actually related.

This paper reports the results of these comparative trials.

MATERIALS AND METHODS.—The original sources of the viruses were from commercial pepper fields located near Canal Point, Florida. The viruses were maintained in tobacco, *Nicotiana tabacum* L. var. Turkish, for the mechanical transmission tests (except that California Wonder pepper was used for certain dilution-end-point trials) and in California Wonder pepper and cucumber, *Cucumis sativus* L. var. Marketer, for the tests on aphid transmission.

Mechanical inoculations were made with freshly expressed sap rubbed by means of cotton swabs onto leaves that had been dusted with 500-mesh Carborundum. All plants were rinsed with tap water immediately after the inoculations had been completed.

Green peach aphids were colonized on caged California Wonder pepper plants. Cotton aphids were reared on leaf cuttings of celery in moist chambers (5). All aphid transfers were made by means of camel's-hair brushes. The insects were starved in glass shell vials, and feedings were timed with a stopwatch. Late-instar or adult apterae were used in all cases. Plants were fumigated with nicotine before being placed in the greenhouse and were routinely sprayed to control insects.

All plants were grown in an air-conditioned greenhouse in which the temperature ranged from 75°–80°F.

IDENTIFICATION OF THE VIRUSES.—*Host range.*—Three to 5 plants of a selected group of plant species were inoculated with each virus isolate at each time. The total number of plants tested varied from 10–25 depending on symptom expression.

Systemic symptoms were produced by each of the 3 viruses in the following species: CHENOPODIACAE—swiss chard (*Beta vulgaris* L. var. *cicla*); COMPOSITAE—zinnia (*Zinnia elegans* Jacq.); CUCURBITACEAE—cucumber (*Cucumis sativus* L. var. Marketer) and balsam vine (*Momordica charianta* L.); SOLANACEAE—eggplant (*Solanum melongena* L.), nightshade (*S. gracile* Link), pepper (*Capsicum frutescens* L. var. California Wonder, Tabasco, and Yolo Wonder), *Physalis peruviana* L., tobacco (*Nicotiana tabacum* L. var. Turkish), and *N. glutinosa* L.

Symptoms were produced in the following species by SCMV but not by PCV or POLV: COMMELINACEAE—wandering jew (COMMELINA sp.) and UMBELLIFERAE—celery (*Apium graveolens* var. *dulce* (Supreme Golden).

Symptoms were not produced by any of the 3 viruses in the following species: COMPOSITAE—aster (*Callistephus chinensis* Nees var. Crego); CRUCIFERAE—cabbage (*Brassica oleraca* L.); LEGUMINOSAE—alfalfa (*Medicago sativa* L.), cowpea (*Vigna sinensis* var. Blackeye), string bean (*Phaseolus vulgaris* L. var. Black Valentine), and lima bean (*P. limensis* L. var. Fordhook).

Symptomatology.—The symptoms caused by the 3 isolates were generally similar in most species of plants tested (Table 1); those caused by SCMV were most severe and those caused by PCV the mildest.

TABLE 1.—*Symptoms in certain plant species caused by 3 strains of cucumber mosaic virus*

Family and species	Symptoms[a] caused by		
	SCMV	POLV	PCV
Chenopodiaceae			
Beta vulgaris var. *cicla*	VC, SM, SS	VC, MM, SS	CR
Commelinaceae			
Commelina sp.	VC, MM, CR	None	None
Compositae			
Zinnia elegans Jacq.	VC, SM	VC, SM	VC, MM
Cucurbitaceae			
Cucumis sativus L.			
var. Marketer	LC, SS, SM, N, D	LC, SM	YD
Momordica charianta L.	LC, N, SS	LC, N, SS	LC, N, SS
Solanaceae			
Capsicum frutescens L.			
var. California Wonder, Yolo Wonder, and Tabasco	VC, LC, SS, RS	VC, NOL	VC, LC, CM
Nicotiana tabacum L.			
var. Turkish	VC, SM	VC, SM	VC, MM
N. glutinosa L.	VC, SM, SS	VC, SM	VC, MM
Physalis peruviana L.	VC, SM	VC, SM	VC, SM
Solanum gracile Link.	VC, MM	VC, MM	VC, MM
S. melongena L.	VC, LC, SM, SS	VC, LC, MM	VC, MM
Umbelliferae			
Apium graveolens L.			
var. *dulce* (Supreme Golden)	VC, LRS, MM, SM, OL	None	None

[a] The letters indicate the following symptoms: CM, calico mottle; CR, chlorotic rings; D, death at times; LRS, local red spots; MM, mild mottle; N, necrosis; NOL, necrotic oak-leafing on senescent leaves; OL, oak-leafing; RS, red-spider-like injury; SS, severe stunting; SM, severe mottle; VC, vein clearing; and YD, yellow dots. Order of use of letters indicates order of appearance of symptoms.

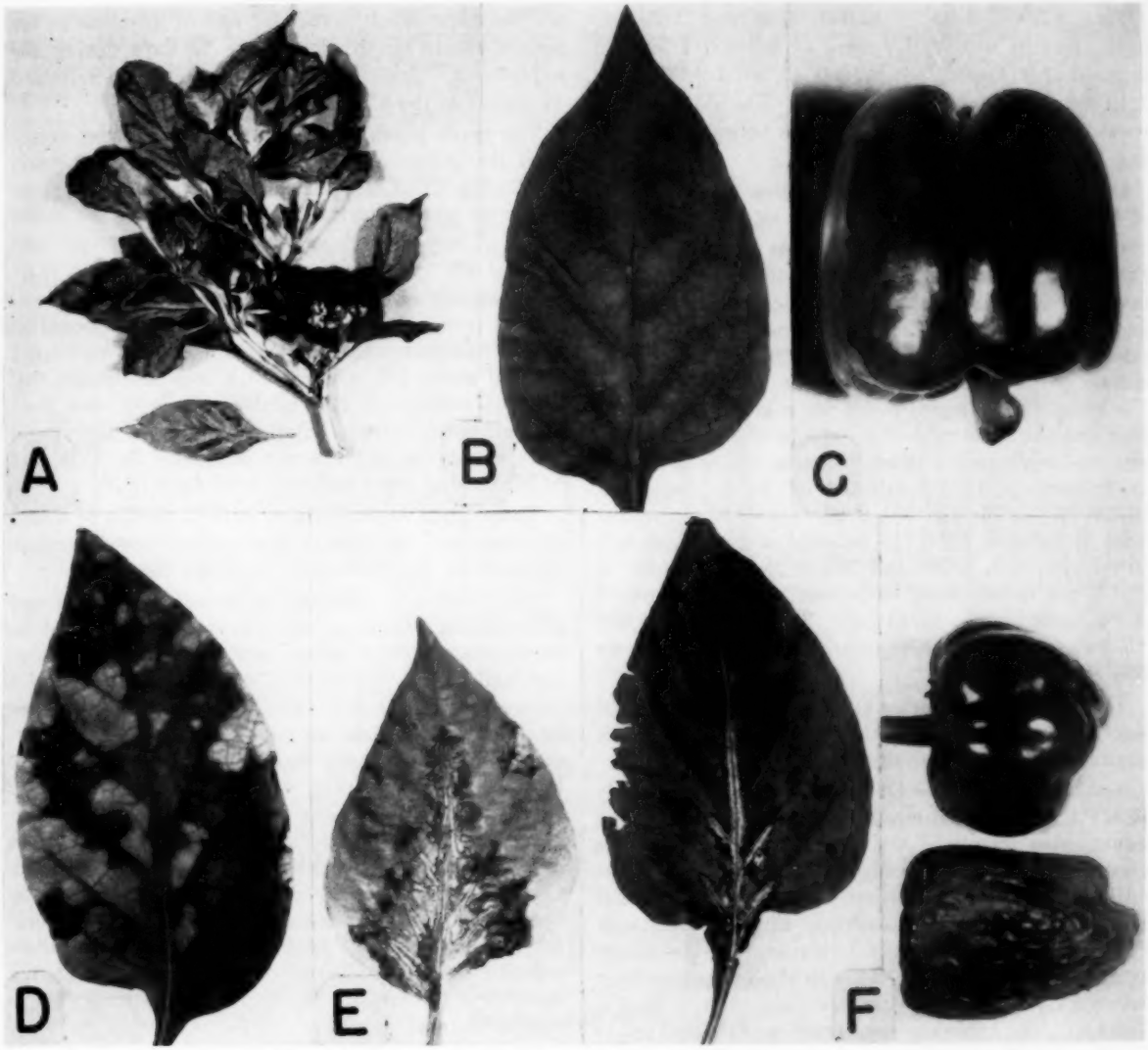

FIG. 1. Symptoms of cucumber mosaic virus strains on California Wonder pepper. A) Symptoms caused by southern cucumber mosaic strain. Note stunting of the plant, etiolation of the leaf (lower), and flattening of the pod. B) Healthy leaf. C) Healthy fruit. D) Foliage symptoms caused by pepper calico virus. E) Symptoms on lower leaves caused by pepper oak-leaf virus. F) Fruit symptoms caused by pepper oak-leaf virus.

Symptoms usually appeared within 1 week of inoculation. The reactions caused by the 3 viruses in pepper plants are shown in Figure 1.

Cross-protection tests.—Since the symptoms of the 3 isolates varied considerably in pepper, cucumber, and *N. glutinosa* it was possible to try cross-protection tests to determine the degree of relationship among the 3 viruses. Plants of cucumber, pepper, and *N. glutinosa* infected with PCV were inoculated with SCMV or POLV. Usually at least 10 plants were inoculated with a challenge virus (SCMV or POLV) and the trials were repeated several times. Inoculations were made by rubbing leaves that had been inoculated with the first virus as well as those that had not. In all cases the challenge virus (SCMV or POLV) failed to produce symptoms, indicating that the 3 viruses are immunologically related and are probably strains of cucumber mosaic virus.

Physical properties.—California Wonder pepper was used as both the source of inoculum and test plant in determining the dilution end point of SCMV and PCV; Turkish tobacco was used for POLV. Freshly expressed sap of plants that had been diseased for 9–14 days was used as inoculum. Plants were inoculated in groups of 12 (pepper) or 6 (tobacco), and 4 (pepper) or 5 (tobacco) replications were carried out. Results with SCMV were as follows: 1:500, 21 infected of 48 inoculated; 1:1000, 16/48; 1:5000, 9/48; 1:10,000, 6/48; and 1:20,000, 0/48. Results with PCV were as follows: 1:500, 21 infected of 48 inoculated; 1:1000,

13/48; 1:5000, 3/48; 1:10,000, 4/48 and 1:20,000, 0/48. Results with POLV were as follows: 1:500, 28 infected of 30 inoculated; 1:1000, 23/30; 1:5000, 8/30, 1:10,000, 2/30 and 1:20,000, 0/30. The dilution end point for all 3 viruses seemed to lie between 1:10,000 and 1:20,000.

In tests on the thermal inactivation point of each of the 3 strains of virus, Turkish tobacco was used both as a source of inoculum and as the test species. Freshly expressed sap from plants that had been diseased for 9–14 days was used as inoculum. Two ml of undiluted sap was placed in cotton-stoppered test tubes and heated in hot water for 10 minutes (plus 1 minute to allow the juice to come to temperature). The tubes were plunged in ice water at the end of this time and the juice rubbed on groups of 6 plants. The test was replicated 4 times. Results for SCMV were as follows: 55°C, 22 infected of 24 rubbed; 60°, 18/24; 65°, 5/24; and 70°, 0/24. For PCV the results were as follows: 55°C, 19 infected of 24 inoculated; 60°, 11/24; 65°, 3/24; and 70°, 0/24. In the case of POLV the results were as follows: 55°C, 18 infected of 24 rubbed; 60°, 13/24; 65°, 2/24; and 70°, 0/24. All 3 virus strains became inactivated at a temperature between 65° and 70°C.

The figures for dilution end point and for thermal inactivation point are within the range expected for strains of cucumber mosaic virus.

Aphid transmission.—Of the 3 strains of virus, only SCMV had been used in other than very limited insect transmission trials. SCMV had been characterized as a nonpersistent aphid-borne virus (5), and there was little reason to assume that the other strains did not have similar vector relationships. Limited trials with PCV, however, indicated it is transmitted less easily by aphids than is SCMV. Also, field observations have shown PCV to be much less prevalent than is SCMV. Therefore, the following experiment was carried out to obtain more information on the vector-virus relationships of these 3 virus strains.

Seedlings of pepper and of cucumber were inoculated with each of the 3 viruses. The following week 1 plant of each species for each virus strain (6 plants in all) was selected for use as an inoculum source plant. Cotton and green peach aphids were taken from stock colonies and fasted for approximately 1 hour. One aphid of each species was then given an uninterrupted acquisition feeding period of 20 seconds or less (if the aphid raised her rostrum before 20 seconds had elapsed, this time was used) on one of the 6 inoculum plants. The aphids were then placed singly on either a healthy pepper or cucumber test plant for a test feeding period of approximately 1 hour. Thus the experiment was factorial, consisting of 2 aphid species, 2 species of inoculum plants, the same 2 species as test plants, and 3 strains of virus, a total of 24 combinations. The entire operation was repeated 35 times over a period of 7 weeks. New inoculum sources were prepared each week throughout the experiment so that the age of infection in the source plants ranged from 7 to 12 days during the experiment. Statistical significance was determined by means of the Chi square test.

The green peach aphid proved to be a better vector than the cotton aphid regardless of the virus strain used (Fig. 2). The ratio of transmission between the 2 species was about 2:1 in favor of the green peach aphid. These differences were statistically significant at the 1 per cent level.

Regardless of the strain of virus used, pepper was a more potent source of inoculum than was cucumber. The differences with SCMV and POLV were at a ratio of about 2:1, whereas PCV was not transmitted from cucumber—all 22 infections obtained came from pepper source plants. Statistically, these differences were significant at the 1 per cent level for PCV and SCMV and at the 5 per cent level for POLV.

The relative susceptibilities of the 2 species of plants (pepper and cucumber) was approximately equal. Statistically, the differences were not significant.

The previously reported phenomenon of increased transmission where an efficient vector was used in combination with a potent source species, over that obtained with a less efficient vector and a less potent source species (5), was again apparent. It was demonstrable for all 3 virus strains (Fig. 2), and the differences were statistically significant at the 1 per cent level. Treatments that utilized the poorer vector (cotton aphid) and less potent source (cucumber) gave many fewer infections than were expected. These differences were also significant at the 1 per cent level. Combinations that were intermediate, i.e. the better vector and poorer source species or the poorer vector and better source species, yielded intermediate amounts of transmission. These results were not significantly different from the anticipated transmission values.

Discussion.—At this time it is not possible to explain, in the case of PCV, the failure of cucumber as a source of inoculum for aphids. It may be that the virus titre in cucumber is too low, but this seems somewhat doubtful since both inoculated and noninoculated leaves are good sources for mechanical inoculation. Another possibility is the existence of a compatibility factor among the host plant, the vector, and the virus that makes recovery very difficult. During the past few years some evidence that supports this hypothesis has accrued (5,7). A somewhat similar situation has been reported from Great Britain (3), where attempts to transmit potato virus C (a strain of potato virus Y) with aphids have all proved negative.

It would seem that the cumulative responses obtained with different combinations of vectors and source species are the result of independent actions acting in simple additive or subtractive combinations. This is most apparent for the SCMV and POLV strains. In the case of SCMV, the deviation from the mean transmission level (23.9 per cent) when the green

peach aphid was used was +8.2 per cent. The summation of the figures (23.9 + 8.2 + 8.2) gives an expected level of transmission of 40.3 per cent, and the observed value was 42.8 per cent. The amount of infection obtained for the converse situation with the cotton aphid was − 8.2 per cent; for cucumber as a source species, it was also − 8.2 per cent. The addition of these 2 negative values to the mean of 23.9 per cent gives an expected transmission level of 7.5 per cent, whereas the actual figure obtained was 10.0 per

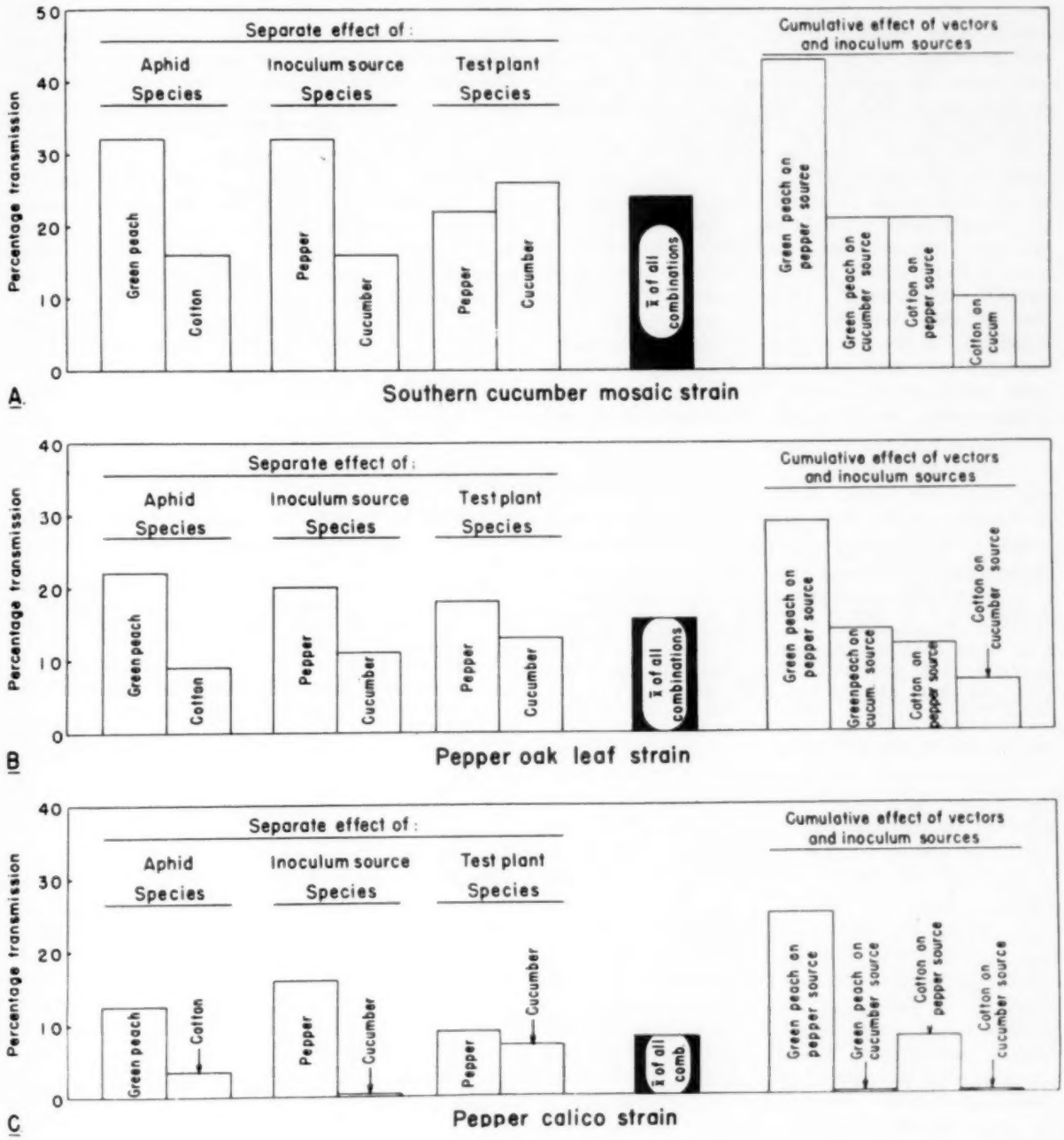

FIG. 2. Results of aphid transmission trials with 3 strains of cucumber mosaic virus, showing (left 3 pairs of columns) the differences among aphid species, inoculum source plants, and test species in terms of total percentage of transmission obtained. The center (dark) bar represents the mean transmission level. The 4 bars on the right show the effect on transmission of pooling various aphid species and inoculum source pairs. The effect of test plant species is not shown, becaue the 2 species apparently were equally susceptible. A) Southern cucumber mosaic virus. B) Pepper oak leaf virus. C) Pepper calico virus.

cent. Similar results can be calculated for POLV. This trend is not as evident for PCV, probably because of the completely negative character of cucumber as a source plant.

The variation in amount of transmission obtained suggests that the choice of vector species and inoculum source plant species can be very important in aphid transmission work. Conceivably, the combination of an inefficient vector, a poor source species, old inoculum source plants, and relatively small numbers of test plants could lead to failure to transmit a virus such as PCV and thus result in an erroneous conclusion that a virus is not aphid borne.

Just how much correlation exists between the poor aphid-transmissibility of PCV and its relative scarcity in the field cannot be stated precisely, although the relationship could certainly exist. Since the virus has not been recovered from a naturally infected weed, it may be that the lack of suitable wild hosts limits its spread. One observation unfavorable to this idea is that the virus does not spread rapidly once it is established in the pepper field. With SCMV, within-field spread is quite rapid, often occurring over large areas within several months.

Apparently, several strains of cucumber mosaic virus exist in Florida. The most common strain would seem to be the one here called southern cucumber mosaic virus (SCMV), which is similar to strains reported as occurring in central Florida by Wellman (9) and Anderson (1).

Townsend and Felix (8) and Felix (4) have reported a pepper virus that may be similar to either the POLV or PCV strains described here. It also might be noted that Felix (4) has reported several types of symptoms on celery in this area and tentatively concluded that different viruses were involved. The author has found a similar diverse range of symptoms in celery inoculated with SCMV and is of the opinion that there is but 1 virus involved.

Anderson (2) has recently described the aster-ringspot virus from central Florida. In certain respects, this virus resembles cucumber mosaic virus. It was not transmitted by means of aphids but causes symptoms on pepper somewhat similar to those caused by PCV. Perhaps, as with PCV, certain rather specific combinations of host plant and vector species are necessary to transmit it with any degree of regularity.

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METHODS FOR ESTIMATING NUMBERS OF SOIL MICROORGANISMS LYTIC TO FUNGI[1]

H. Paul Carter and John L. Lockwood[2]

SUMMARY

When an agar medium containing a 2- to 4-day-old fungus culture prepared by seeding the agar with conidia was sprayed with a soil suspension, colonies of soil microorganisms surrounded by lytic zones appeared on the agar in from 2 days to 3 weeks. These organisms were predominantly members of the genus *Streptomyces*. The number of microorganisms lytic to fungi obtained by this method was found to depend on a number of variables. Of several fungus-agar combinations tested, the greatest number of colonies appeared, and zones were most distinct, on peptone agar containing *Glomerella cingulata*. Other variables affecting numbers of lytic colonies obtained were method of seeding of test fungus (spores mixed with or on the surface of the agar), amount of agar per petri plate, soil dilution, number of days between seeding and addition of soil, and number of days between addition of soil and taking of data. A number of interactions involving these variables were statistically significant.

The potential importance of lytic microorganisms in the ecology of soil fungi, particularly soil-borne plant pathogens, suggested a need for a method of analyzing soils for microorganisms capable of lysing fungi. Other aspects of lysis of fungi by soil microorganisms and fungicides are the subject of another report.[3]

GENERAL METHOD.—An agar medium that contained conidia of a suitable fungus was placed in sterilized petri plates. After the appearance of abundant mycelium, usually in 2–3 days, the agar in the plates was sprayed with a soil suspension. Two days to 3 weeks later, colonies of microorganisms developed on the surface of the agar. Occasional colonies were surrounded by clear areas free from mycelium of the test fungus, an indication that such colonies had lysed the test fungus.

Soil samples were collected at a depth of 2 in. and were of 2 types, Wooster silt loam and muck. Both were collected near Wooster, Ohio. One g of soil was placed in 100 ml of sterile distilled water and mixed for 1 minute with a mechanical stirrer. Dilutions of 1:1000 and 1:10,000 were prepared, and approximately 0.6 ml of suspension was sprayed on the agar in each petri plate. If more of the suspension was used, bacteria obscured the lytic zones. Spraying was done with an atomizer (DeVilbiss No. 15) operated by compressed air at 10 lb./sq. in. The nozzle was fastened 40 cm above the plates. A cotton filter was placed in the air line, and the entire apparatus was autoclaved prior to each experiment. Cultures were incubated at 26°C.

MICROORGANISMS CAUSING LYSIS.—During the development of the method for analyzing soils for the presence of microorganisms lytic to fungi, several thousand such organisms were isolated. Of these, over 100 were selected and cultured. These organisms were almost all members of the order Actinomycetales and belonged to the genus *Streptomyces*.[4] A few bacteria possessing lytic properties were isolated. Most colonies developed very slowly on the various substrates, not appearing until 1–3 weeks after application of soil suspensions to the medium. A few colonies developed rapidly, with the appearance of zones the second day following application of soil suspensions.

QUANTITATIVE STUDIES.—By means of the method described above, tests were made of the effects of several variables on the number of lytic colonies that appeared. The variables studied were test fungus, method of seeding test fungus, type of medium, amount of agar per petri plate, soil dilution, number of days between seeding and application of soil suspension, and number of days between application of soil suspension and taking of data. Data were analyzed by analysis of variance. Since few lytic zones developed per plate, data were transformed for the analyses. The conversion factor used was $\sqrt{x} + \sqrt{x+1}$, where x represents the average number of colonies per plate.[5]

Test fungus.—Lytic zones were produced when the agar substrate contained any of the following fungi: *Glomerella cingulata* (Ston.) Spauld. & Schrenk, *Helminthosporium sativum* Pam., King, & Bakke, *Colletotrichum circinans* (Berk.) Vogl., *Cladosporium cucumerinum* Ell. & Arth., *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans., *Rhizoctonia solani* Kühn, *Alternaria solani* (Ell. & G. Martin) Jones &

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Grout, *Colletotrichum lagenarium* (Pass.) Ell. & Halst., and *Verticillium albo-atrum* Reinke & Berth. No lytic microorganisms were obtained when *Fusarium solani* (Mart.) Appel & Wr., *Pythium aphanidermatum* (Edson) Fitzp., or *Saccharomyces cerevisiae* Hansen var. *ellipsoideus* (Hansen) Dekkr. were used.

The first 6 fungi listed were used in further quantitative tests. Spore suspensions from potato-dextrose agar (PDA) cultures (per liter: infusion from 200 g potatoes; glucose, 20 g; agar, 20 g) were prepared at the rate of 20,000 spores per ml. In the case of *R. solani*, a mycelial fragment was transferred to each plate.

The number of zones that appeared on media that contained *G. cingulata* was, in almost all cases, higher than the number that appeared when other fungi were used as the test organisms (Table 1). At the low soil dilution (1:1000), the number of lytic organisms obtained on plates containing *G. cingulata* was usually several times higher than the number obtained on plates containing other test fungi. The numbers of colonies obtained with the different soil dilutions usually differed most widely when *G. cingulata* was used. The greatest number of lytic zones was produced on each of the 3 types of media when *G. cingu-*

TABLE 1.—*Mean number of lytic colonies per plate obtained from soil in factorial experiments in which petri plates containing either of 2 amounts of several agar media were seeded with different fungi, either on the surface or mixed with the agar, then sprayed at various times with either of 2 dilutions of different kinds of soil, with readings made after different intervals*

Variable tested	Mean no.[a] of lytic colonies obtained in 4 experiments			
	3×2×2×2×2 Factorial[b]	2×2×2×2×2 Factorial[c]	6×3×2×2 Factorial[d]	3×3×2×2×2 Factorial[e]
Test fungus				
Glomerella cingulata	7.4**		2.4**	4.8**
Helminthosporium sativum	2.3		0.1	1.0
Colletotrichum circinans	3.5		0.7	0.7
Fusarium oxysporum f. *lycopersici*			0.0	
Cladosporium cucumerinum			0.3	
Rhizoctonia solani			0.2	
Method of seeding test fungus				
Surface of agar		4.3**		
Mixed with agar		10.3		
Type of medium				
Peptone agar			1.6**	4.6**
Yeast agar			0.1	1.6
Soil agar			0.1	0.4
Amount of agar				
10 ml	4.9**	7.5		
15 ml	3.9	7.2		
Type of soil				
Silt loam		3.7**	0.5	1.2**
Muck		10.9	0.8	3.2
Soil dilution				
10^{-3}	6.4**	12.6**	0.9	2.5*
10^{-4}	2.4	2.0	0.4	1.8
Days between seeding and addition of soil				
2	3.2**			
7	5.6			
Days between addition of soil and taking of data				
13	3.6**			
18	5.2			

[a] The single and double asterisks indicate that the *F* for the variable indicated was statistically significant at the 0.05 and 0.01 levels, respectively.

[b] Method of seeding test fungus, mixed with agar; type of medium, peptone agar; type of soil, silt loam. Six plates were used for each treatment. Significant interaction at 0.05 level: soil dilution × days between addition of soil and taking of data. Significant interactions at 0.01 level: soil dilution × amount of agar, soil dilution × test fungus, test fungus × days between seeding and addition of soil.

[c] Test fungus, *G. cingulata*; type of medium, peptone agar; days between seeding and addition of soil, 3 days; days between addition of soil and taking of data, 14 days. Four plates were used for each treatment. Significant interactions at 0.01 level: method of seeding test fungus × soil dilution; soil dilution × type of soil.

[d] Method of seeding test fungus, mixed with agar; amount of agar, 15 ml; days between seeding and addition of soil, 3 days; days between addition of soil and taking of data, 17 days. Four plates were used for each treatment. Significant interactions at 0.01 level: test fungus × type of soil; soil dilution × type of medium; soil dilution × test fungus, type of medium × test fungus; higher order interactions.

[e] Method of seeding test fungus, mixed with agar; amount of agar, 15 ml; days between seeding and addition of soil, 2 days; days between addition of soil and taking of data, 15 days. Three plates were used for each treatment. Significant interaction at 0.05 level: type of soil × test fungus; significant interaction at 0.01 level: type of soil × type of medium.

lata was used as the test fungus. Differences between soil types, with respect to number of lytic zones obtained, were largest when *G. cingulata* was used. These results indicated that *G. cingulata* was a more sensitive test organism than the other fungi tested; moreover, the zones were invariably more distinct and easier to identify with this fungus than with any other. *G. cingulata* also sporulated rapidly and abundantly in culture.

Transfers were made from over 100 colonies around which zones formed, irrespective of the fungus species in the substrate or the type of medium on which they occurred. These isolates were cultured on yeast-extract-glucose agar slants (per liter: yeast extract, 2 g; glucose, 10 g; agar, 20 g). Tests were made to determine if lytic isolates obtained by use of other fungi would also lyse *G. cingulata*. In every case *G. cingulata* was lysed.

Method of seeding test fungus.—Conidia were applied to media by 2 methods: 1) spraying the spore suspensions on the surface of sterile media in petri plates by the technique outlined above, and 2) mixing the spore suspensions with sterile media at approximately 40° C and immediately pouring the mixture into sterile petri plates. Spore suspensions were added to media at the rate (by volume) of 1 part suspension to 5 parts medium.

More lytic colonies were obtained when conidia of *G. cingulata* were mixed with the agar than when they were applied to the surface of the agar; moreover, the number of zones obtained with different soil dilutions differed more widely with the mixed-seeding method than with the surface-seeding method (Table 1). Zones also were more distinct when the mixed method of application was employed.

Type of medium.—In preliminary tests, several media were used for obtaining lytic microorganisms from the soil. Zones occurred only on media that supported appressed growth of the test fungus. Three media, each supporting sparse growth, were used in quantitative tests. These were 1) peptone agar (per liter: peptone, 5 g; agar, 20 g), 2) yeast-extract agar (per liter: yeast extract, 2 g; agar, 20 g), and 3) soil-extract agar (per liter: extract from 20 g soil; agar, 20 g). Soil extract was obtained by autoclaving 20 g of Wooster silt loam in 500 ml of water for 15 minutes at 15 lb./sq. in. The suspension was then centrifuged to remove soil particles.

Plates with peptone agar contained a greater number of lytic colonies than did those with other media (Table 1). The combination of peptone agar and *G. cingulata* produced more lytic zones than did any other fungus-agar combination tested. In 1 of 2 experiments, the numbers of colonies obtained with different soil dilutions differed most widely with peptone agar. Also, differences in results with the 2 soil types were greatest when peptone agar was used.

Amount of agar per petri plate.—Ten-ml and 15-ml amounts of agar were added to plates by use of a measuring syringe (Becton-Dickinson model G6473) that delivered 5 ml per stroke. In 1 experiment (Table 1), significantly more lytic zones were obtained on plates containing 10 ml of agar than on those containing 15 ml. This difference in numbers was largest at the soil dilution 1:1000. In another experiment, these effects were not statistically significant. Since media dehydrated to some extent, 15 ml per plate was usually employed.

Soil dilution.—In general, more colonies of lytic organisms were obtained with soil dilutions of 1:1000 than with those of 1:10,000, but in no case were the differences in numbers proportional to the differences in dilutions (Table 1). The numbers of lytic organisms obtained at the 2 dilutions rarely differed more than 2-fold. When other factors were varied, greater differences in the numbers of lytic zones were obtained when a 1:1000 soil dilution was used than when one of 1:10,000 was used. Significant interactions occurred between soil dilution and all other variables tested except that of days between addition of soil and taking of data.

Days between seeding and application of soil suspension.—Soil suspensions were applied to agar 2 days or 7 days after application of conidia of the test fungus. The same soil sample, refrigerated at 4° C, was used in preparing both sets of plates.

More lytic zones appeared when media were sprayed with soil suspensions 7 days after seeding of the test fungus than when they were sprayed 2 days after seeding (Table 1). Numbers of lytic microorganisms appearing on plates sprayed at 2 and 7 days differed more widely when *G. cingulata* or *H. sativum* were used as test organisms than when *C. cucumerinum* was used.

Days between application of soil suspension and taking of data.—The number of colonies of lytic organisms per plate was determined 13 days and 18 days after media were sprayed with soil suspensions. The number of colonies appearing at 18 days was greater than the number at 13 days after spraying (Table 1). At 18 days, there was a greater difference in colony numbers at the 2 soil dilutions than was obtained at 13 days.

DISCUSSION.—The results obtained indicate that the apparent number of mycolytic organisms in a soil sample depends on several variable factors. The occurrence of a number of statistically significant differences within each variable and of significant interactions between variables indicates that standardized procedures are essential for estimating numbers of lytic microorganisms. The large number of significant interactions also provides a basis for choosing optimum conditions for maximum sensitivity of the assay. The set of conditions found most satisfactory for estimating numbers of soil microorganisms possessing lytic properties in these investigations was as follows: test fungus, *Glomerella cingulata;* method of seeding test fungus, spores mixed with agar; type of

medium, peptone agar; amount of agar per petri plate, 10 or 15 ml; soil dilution, 1:1000; days between seeding and application of soil suspension, approximately 7 days; days between application of soil suspension and taking of data, approximately 18 days. The temperature at which cultures were incubated was 26°C. The methods described in this paper should be useful for estimating populations of mycolytic microorganisms in soils or other media.

The lytic method might also have possibilities as a screening tool in searching for antifungal antibiotics. It is probable that a part of the lytic action of *Streptomyces* isolates was due to the production of antifungal antibiotics. Such antibiotics were lytic to fungi under conditions similar to those described in this paper.[3] Contamination was rarely a problem with the lytic method, since the fungus-agar substrate favored those organisms capable of lysing the fungus.

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LYSIS OF FUNGI BY SOIL MICROORGANISMS AND FUNGICIDES INCLUDING ANTIBIOTICS[1]

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SUMMARY

Several species of fungi growing on agar media were lysed by the action of certain isolates of *Streptomyces* spp. from soil. Antibiotics and fungicides that inhibited growth of *Glomerella cingulata* also lysed mycelium of this fungus on peptone agar. Lytic zones produced by the colonies of *Streptomyces* spp. were usually clearer than those resulting from the action of antibiotics and fungicides. Some lytic zones produced by *Streptomyces* spp. were entirely free from mycelium of the test fungus. The *Streptomyces* spp. lysed both living and dead mycelium of *G. cingulata* in peptone agar, but antibiotics and fungicides lysed only living mycelium. Mycelium of *G. cingulata* growing in peptone broth was partially disintegrated when Acti-dione or isolate 55 of *Streptomyces* was added to the cultures.

Although there have been frequent reports of inhibition of fungi by microorganisms and by fungicidal chemicals, lysis of fungi has been observed infrequently. Disintegration of fungal mycelium by actinomycetes has been observed in culture (12, 15, 17), and several instances of lysis of fungi by bacteria are known (1, 6, 7, 9, 10, 11, 13). Lysis of fungal mycelium in contact with soil also has been observed, but the agents responsible for such action were not stated (2, 4, 5). Certain plant diseases have been controlled experimentally with mycolytic microorganisms (2, 5, 7, 10, 11, 13).

In work by Leben and Lockwood (unpublished data), contaminating microorganisms that sometimes appeared on agar plates used for assaying antifungal antibiotics were frequently surrounded by zones in which the mycelium of the test fungus had disintegrated. As a result of these observations, investigations were made of the lytic action on the fungus *Glomerella cingulata* (Ston.) Spauld. & Schrenk of various microorganisms and of several fungicides including antibiotics.

The modes of action of isolates of *Streptomyces* spp., of antibiotics, and of fungicides as agents of lytic activity are not known. In this paper, therefore, such terms as "lytic microorganism" and "lytic agent" are used when referring to microorganisms or substances that initiate lysis in fungi or are themselves the causal agents of such disintegration.

Materials and methods.—*Method of testing soil microorganisms and fungicides for lytic activity.*—Soil microorganisms used were obtained during the development of a method for isolating lytic microbes from soils (3). Microorganisms lytic to mycelium of *Glomerella cingulata*, *Colletotrichum circinans* (Berk.) Vogl., or *Helminthosporium sativum* Pam., King. & Bakke were employed.

All lysing organisms were members of the genus *Streptomyces*. They were cultured on yeast-extract agar (per liter: yeast extract, 2 g; glucose, 10 g; agar, 20 g) at 26°C. Sixteen antibiotics, primarily of an antifungal nature, and 5 other fungicides were

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used in the investigations. Each was dissolved in an appropriate solvent in concentrations of 1000, 500, 250, and 125 ppm of active material. The fungicides were filtered to remove any insoluble material present.

Agar tests for lytic activity were made in Petri plates on peptone agar (per liter: peptone, 5 g; agar, 20 g) containing a culture of the fungus *G. cingulata*. The culture was prepared by mixing a conidial suspension (containing approximately 20,000 spores per ml of water) with the warm agar at the rate of 1 to 5, respectively, and then incubating the mixture for 2–3 days. Chemicals or spore suspensions of the lytic isolates of *Streptomyces* spp. were applied to the surface of the agar in sterile blotting paper disks (diameter, 6 mm) that contained 0.02 ml of solution or suspension. Duplicate disks were prepared and placed on different plates. Chemicals were tested for inhibition of *G. cingulata* by a similar method except that disks containing the chemicals were applied immediately after plates were poured and the agar hardened. Pyridine, the solvent for ascosin, produced a narrow lytic zone; therefore disks containing pyridine were dried before being placed on the agar. Other solvents were neither lytic nor inhibitory.

For tests made in liquid media, 60 ml of peptone broth (per liter: peptone, 5g) were added to each of a series of 125-ml flasks. Ten ml of a conidial suspension of *G. cingulata* containing approximately 20,000 spores per ml was added to each of the flasks. After *G. cinculata* had grown for 5 days in stationary culture, either Acti-dione dissolved in ethanol or a spore suspension of isolate 55 of *Streptomyces* spp. was added to the cultures. Cultures were incubated for an additional 5 days. Dry weights of mycelium were determined at the time of addition of the lytic agents and, in treated and control flasks, at the end of the incubation period. Ethanol, at the highest concentration used (3500 ppm), did not lyse *G. cingulata*.

Photometric method for determining lysis.—A method similar to that used by Hollis (8) was devised for determining changes in apparent density of mycelium. The apparatus consisted of a microscope with substage condenser, diaphragm, and concave mirror but with eyepiece and objectives removed; a microscope lamp with condenser and diaphragm; and an electrophotometer (Photovolt model 200M). The apparatus was adjusted to give readings of 100 when a beam of light 4 mm in diameter at stage level was passed through petri plates with peptone agar only and readings of approximately 30 when the light was passed through agar that contained dense mycelium. It was necessary that all adjustments be kept constant. One reading was made for each zone each time data were taken, and all readings for a given zone were taken in the same position on the plate. An increase in the amount of light transmitted indicated that lysis had occurred. Lysis of mycelium of *G. cingulata* was perhaps greater than indicated by the photometric readings since the agar became more opaque as dehydration increased.

All experiments were repeated 1 or more times with similar results.

RESULTS.—*Evidence for lytic activity.*—Zones produced by the addition of chemicals or of certain isolates of *Streptomyces* spp. to agar containing a culture of the fungus *Glomerella cingulata* were studied by microscopic examination and with an electrophotometer. The mycelium in these zones appeared to be less dense when examined with the naked eye than did that in the agar surrounding the zones. Microscopic examination of mycelium in the zones was made by direct observation of the agar in the petri plates and of small pieces of agar cut from the zones and crushed on glass slides. Anilin blue or neutral red were sometimes used to stain mycelium. Hyphal threads from all lytic zones examined were less distinct and fewer in number than in control plates. No mycelium could be seen in zones produced by certain isolates of *Streptomyces* spp.

The amount of light transmitted through certain zones was compared with the relative amount of mycelium observed microscopically in the same zones. Those zones through which the most light was transmitted contained fewer and less distinct hyphae than did those through which the least light was transmitted.

Continued growth of the test fungus outside the zone following treatment of an agar culture with a chemical or with certain isolates of *Streptomyces* spp. made it difficult to determine whether the zone produced was merely the result of the prevention of additional growth of the fungus in the zones or was due to the actual dissolution of the mycelium. By use of the photometer, the amount of light transmitted through such zones was found to increase from day to day. This effect, therefore, was interpreted as lytic. The percentage light transmission through agar containing mycelium of *G. cingulata* that was not treated with chemicals or with isolates of *Streptomyces* spp. decreased daily until maximum growth was achieved.

An antifungal antibiotic, Acti-dione, and a *Streptomyces* isolate, No. 55, both of which appeared to lyse mycelium of *G. cingulata* in agar, were tested for their effect on 5-day-old mycelium of this fungus growing in peptone broth (Table 1). Five days after the addition of Acti-dione to broth cultures of the fungus, dry weights of mycelium from treated cultures were 30–51 per cent less than those of the original mycelium, whereas the dry weights of mycelium from untreated cultures were 23–24 per cent greater than those of the original mycelium. Isolate 55 of *Streptomyces* spp. caused a similar decrease in weight of mycelium. Apparently, the mycelium of *G. cingulata* was lysed by these agents.

Lytic activity of isolates of Streptomyces spp. and of fungicides.—Characteristics of the lytic zones produced by *Streptomyces* spp. were studied by streaking isolates on 2-day-old cultures of *G. cingulata* grow-

FIG. 1. Lytic zones produced by isolates of *Streptomyces* spp. streaked on peptone agar containing a culture of *Glomerella cingulata*.

ing on peptone agar (Fig. 1). Lytic zones developed well only on media low in nutrients that supported appressed growth of the test fungus (3). Detectable zones rarely developed on media that supported abundant aerial growth. Zones usually appeared 2–10 days after streaking. Different isolates varied greatly in size and clarity of zones produced as determined by visual examination. Occasionally, zones were observed that extended less than 1 mm from the colony of *Streptomyces* spp. Conversely, some colonies produced zones that covered the greater part of a petri plate. The same isolate always produced zones of the same relative size and clarity. In general, the narrower the zones the greater was their visual clarity, and any given zone appeared uniform throughout with respect to density of mycelium. In some zones no mycelium could be detected by microscopic examination of stained or unstained preparations. Zones produced by 2 different colonies of *Streptomyces* spp. in the same plate sometimes overlapped. These overlapped areas frequently contained less mycelium of the test fungus than did zones produced by single colonies. The above observations were confirmed by photometric and microscopic determinations. No direct parasitic action of the colonies of *Streptomyces* spp. on the mycelium of *G. cingulata* was observed.

TABLE 1.—*Lytic action of Acti-dione and of isolate 55 of Streptomyces spp. on mycelium of Glomerella cingulata in peptone broth*

Lytic agent	Concentration ppm	Percentage (by weight) of original mycelium remaining 5 days following addition of lytic agent to a 5-day-old culture[a]	
		Test 1	Test 2
Acti-dione[b]	125		70.3
Acti-dione	500	48.6	62.2
Acti-dione	1000	54.3	
Isolate 55		62.9[c]	43.2[c]
None		122.9	124.3

[a] Based on average of 4 cultures.
[b] 3-[2- (3,5-Dimethyl-2-oxocyclohexyl) -2-hydroxyethyl] glutarimide.
[c] Based on combined weight of mycelium of *G. cingulata* and isolate 55 of *Streptomyces* spp.

Diffusible substances apparently were produced by the lytic microorganisms. Disks of agar were taken from areas adjacent to colonies of lytic microorganisms growing on unseeded peptone agar. Following transfer of the disks to agar containing *G. cingulata*, zones that were identical to those that surrounded the lytic microorganisms themselves appeared around some of the disks.

Fungicides were applied to peptone agar containing 2-day-old cultures of *G. cingulata* and to peptone agar freshly seeded with spores of this fungus. From 1 to 5 days following application of chemicals to the fungus-agar substrates, zones surrounded most of the

disks. Of 21 chemicals tested, all except griseofulvin, mycophenolic acid, and neomycin inhibited growth in freshly seeded plates; the same chemicals also lysed *G. cingulata* when applied to 2-day-old cultures (Table 2). Frequentin and gladiolic acid produced narrow lytic zones on 2-day cultures but did not prevent growth of *G. cingulata* in freshly seeded plates. Lysis of *G. cingulata* by all chemicals except frequentin and gladiolic acid began within 24 hours after application; lytic activity by these 2 materials was not observed until 11 days after application. Lytic zones and zones of inhibition produced by the same chemical at a given concentration were of approximately equal diameters and clarity as determined visually. The replacement of assay disks with new disks containing the same fungicide did not increase the amount of light transmitted through the zones. Although lytic zones produced by antibiotics and by fungicides usually appeared sooner than did zones produced by *Streptomyces* spp., in many cases zones produced by the latter were more translucent.

Autolysis of *G. cingulata* in peptone agar was observed (see seeded control in Table 2). The amount of mycelium increased rapidly following application of conidial suspensions of *G. cingulata* to the agar, maximum density being obtained in 2–4 days. Light transmission through the agar then increased gradually for about 10 days to a point at which no further change occurred. The amount of mycelium that disintegrated as a result of autolysis was considerably less than the amount that disintegrated when *Streptomyces* spp. or chemicals were present.

Comparison of mode of action of Streptomyces spp. and of fungicides.—The fact that most of the antifungal chemicals tested lysed mycelium of *G. cingulata* suggested that lytic action of *Streptomyces* spp. was due to antibiotics which they produced. An attempt was made to determine if the modes of action of the chemicals and *Streptomyces* spp. were similar or different. Petri plates containing peptone agar in which *G. cingulata* was growing were exposed to chloroform vapor for 4 hours. Similar plates were autoclaved for 5 minutes at 10 lb./sq. in. Both methods killed *G. cingulata*. Assay disks containing fungicides and spore suspensions of lytic microorganisms were placed on the surface of the medium. Whereas *Streptomyces* spp. lysed both living and dead mycelium (Table 3), the chemicals lysed only living mycelium (Table 2). The action of *Streptomyces* spp. in lysing mycelium of *G. cingulata*, therefore, appeared to differ from that of the fungicides.

More light was transmitted through plates that contained mycelium killed by chloroform or by autoclaving than through plates in which the mycelium was not killed (Tables 2, 3). There appeared to be less mycelium in those plates that were exposed to chloroform than in those that were autoclaved. Chloroform vapor may exert lytic effects on fungi similar to those produced by other chemicals.

TABLE 2.—*Relative amount of lysis by antibiotics and by fungicides of living and dead mycelium of Glomerella cingulata in agar as determined by photometric method*

	Mean percentage light transmission[b]					
			Killed mycelium			
	Living mycelium		Autoclave		Chloroform	
Chemical[a]	0 Days[c]	15 Days[c]	0 Days[c]	15 Days[c]	0 Days[c]	15 Days[c]
Acti-dione	28	58	41	41	62	59
Antimycin A	30	57			60	59
Ascosin	29	70	38	38	64	62
Candicidin	26	60			62	62
Endomycin	29	68			63	62
Fradicin	25	55	39	37	62	60
Frequentin	27	50			63	65
Gladiolic acid	30	55			58	60
Gliotoxin	24	50			72	63
Griseofulvin	23	34			68	70
Helixin B	26	59			62	62
Mycophenolic acid	27	24			67	70
Mycostatin	30	78	44	45	61	61
Neomycin	25	30			65	63
Rimocidin	28	62			70	71
Thiolutin	27	75			59	63
Coromerc[d]	29	58			61	55
Ferbam[e]	30	70			71	68
Mercuric chloride	30	62			63	60
Thiram[f]	27	65	42	40	67	65
Zineb[g]	29	70			67	64
Seeded control	27	36	43	43	65	62
Unseeded control	100	91	100	92	100	90

[a] Appreciation is expressed to the following manufacturers and person for supplying chemicals used in these tests: Commercial Solvents Corp., Olin-Mathieson Corp., S. B. Penick and Co., Charles Pfizer and Co., and Dr. Curt Leben.

[b] A percentage light transmission of 23–30 indicates no lysis; one of 100 indicates complete lysis (or no fungus growth).

[c] Time after application of chemicals to 2-day-old cultures of *G. cingulata* in peptone agar.

[d] *N*-phenylmercuriethylenediamine.

[e] Ferric dimethyldithiocarbamate.

[f] Bis(dimethylthiocarbamoyl) disulfide.

[g] Zinc ethylenebis[dithiocarbamate].

DISCUSSION.—Although lysis of fungi in soils seldom has been reported (2, 4, 5), lytic activity of microorganisms in soils may be an important process in the ecology of soil microflora. It is well known that the population of soil microorganisms is influenced by such factors as soil depth, fertility, moisture, temperature, and cropping sequences (16), but the mechanisms bringing about changes in population are not well understood. Many isolates of *Streptomyces* spp. obtained from soil lysed fungal mycelium growing in a medium deficient in nutrients. This suggests that lytic action may occur in soils, most of which are similarly deficient in nutrients for optimum fungus growth. Furthermore, the capacity to lyse fungus mycelium may confer a competitive advantage on the lytic microbes, for it is possible that the metabolites released by lytic action are utilized by the lytic micro-

TABLE 3.—*Relative amount of lysis by isolates of Streptomyces spp. of living and dead mycelium of Glomerella cingulata in agar as determined by photometric method*

Streptomyces isolate	Mean percentage light transmission[a]					
	Living mycelium		Killed mycelium			
			Autoclave		Chloroform	
	0 Days[b]	15 Days[b]	0 Days[b]	15 Days[b]	0 Days[b]	15 Days[b]
Isolate 3	24	46			64	70
Isolate 12	24	77	51	75	65	80
Isolate 22	32	66				
Isolate 33	36	79			66	80
Isolate 41	29	57			60	75
Isolate 49	31	61	47	74		
Isolate 55	33	78	38	59		
Isolate 56	26	54			62	80
Isolate 64	26	65				
Isolate 69	32	71				
Isolate 76	31	78			60	74
Seeded control	27	36	43	43	65	62
Unseeded control	100	91	100	92	100	90

[a] A percentage light transmission of 24–36 indicates no lysis; one of 100 indicates complete lysis (or no fungus growth).

[b] Time after transferring isolates of *Streptomyces* spp. to 3-day-old cultures of *G. cingulata* in peptone agar.

organisms in growth processes.

Since antibiotics are produced by many species of *Streptomyces*, it might be inferred that lytic action of these isolates was due to antibiotics that they produced. This may be true in part, but *Streptomyces* spp. lysed both living and dead mycelium whereas antibiotics lysed only living mycelium, an indication that other substances also were produced. Saltan (14) observed disintegration of dead bacterial cells by *Streptomyces* spp., presumably as a result of enzymatic action. Enzymes synthesized by lytic microorganisms may, in addition to the antibiotics, be responsible for the disintegration of the mycelium of fungi. The action of antibiotics and other fungicides in lysing fungal mycelium may be a hastening of autolytic processes as a result of poisoning of the mycelium. Since these chemicals are not enzymatic in nature, their action could not be by direct dissolution of mycelium.

Zones produced by different isolates of *Streptomyces* spp. sometimes overlapped when streaked on agar in the same plate. Those areas that overlapped frequently contained less mycelium than did zones produced by the single isolates. This suggests that some isolates produced enzymes that initiated partial disintegration of the mycelium of the test fungus, whereas other isolates synthesized enzymes that lysed other constituents of the mycelium. This explanation would account for the additive effects observed.

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INFLUENCE OF THE STUBBY-ROOT NEMATODE ON GROWTH OF ALFALFA[1]

Ivan J. Thomason and S. A. Sher[2]

SUMMARY

Alfalfa plants of the variety California Common, widely grown in California, were severely stunted when grown in the greenhouse in soil infested with the stubby-root nematode, *Trichodorus* sp. Dry weight of foliage of plants grown in infested soil was significantly less than that of plants grown in noninfested soil. A serious reduction in lateral root development occurred, also, in plants grown in infested soil. Soil fungi that were present in stunted alfalfa roots from Trichodorus-infested soil did not cause reduction in growth of alfalfa.

Trichodorus spp. have been found in association with the roots of numerous crop and ornamental plants in southern California. In several fields, poor growth of alfalfa (*Medicago sativa* L.) has been associated with large numbers of one of these species, the stubby-root nematode.

The stubby-root nematode was first reported to be a pest of vegetable and field crops in the southern United States by Christie and Perry[3] in 1951. They listed a number of crops observed to be injured in the field by this nematode, and showed experimentally that the roots of beets, *Beta vulgaris* L., and of sweet corn, *Zea mays* L., were injured.

In March, 1954, the authors examined soil samples from a field of severely stunted Sudangrass, *Sorghum vulgare* var. *sudanense* Hitchc., near Chino, California. The soil, when washed through screens, contained 200–300 stubby-root nematodes[4] per pint. Preliminary greenhouse fumigation tests with ethylene dibromide resulted in increased growth of alfalfa and Sudangrass in this soil.

Because of the importance of alfalfa in California agriculture and of the apparently widespread distribution of the stubby-root nematode, experimental tests were begun to determine what influence this nematode might have on the growth of alfalfa.

MATERIALS AND METHODS.—Stubby-root nematodes for use in the tests were originally obtained from the field of Sudangrass referred to above. The nematodes were removed from the soil by washing and screening through 32-, 170-, and 325-mesh sieves. From 100 to 300 nematodes were then picked out individually with a bamboo splinter and introduced into pasteurized soil in 3-quart metal pots. Alfalfa seed (var. Africa) was then planted in the pots.

After 6 months' development in the greenhouse, the nematodes were washed and screened from the soil. The nematode suspension was then placed on a modified Baermann funnel[5] for final extraction. The only nematodes recovered besides the Trichodorus were members of the Cephalobidae and Dorylaiminae. The technique outlined above was used in all subsequent tests to extract the nematodes from the soil for determining numbers of Trichodorus per pot. Nematodes recovered were counted with the aid of a dissecting microscope.

Two tests were conducted to determine what influence the stubby-root nematode would have on the growth of alfalfa. Each test consisted of 20 3-quart pots of steam-pasteurized sandy loam planted to alfalfa (var. California Common).

The soil of 10 pots in each test was infested with *Trichodorus* sp. at the time of planting. The suspension of nematodes was introduced into the soil by means of a probe and a pipette. After emergence, the alfalfa was thinned to 7 plants per pot in Test No. 1 and to 2 plants per pot in Test No. 2. Nematodes used for inoculum in Test No. 2 were recovered from the soil used in Test No. 1.

Greenhouse temperatures ranged from 19° to 32°C.

RESULTS.—*Growth of alfalfa in soil infested with Trichodorus sp.*—In each test, oven-dry (100°C for 48 hours) weights of foliage from 3 successive cuttings were obtained. The dry weights from all 3 cuttings were similar and therefore only these from the first 2 cuttings in each test are reported. The dry weight of foliage from plants growing in Trichodorus-infested soil was reduced 32 per cent (Table 1) in Test No. 1. The reduction in dry weight of foliage in the first 2 cuttings was nearly the same. The injury to alfalfa in infested soil was even more severe in Test No. 2 (Fig. 1, A). Dry weight of foliage was reduced 88 per cent below that of plants grown in noninfested soil (Table 1). The reduced foliage production by alfalfa plants grown in infested soil was highly significant statistically.

No typical leaf symptoms were associated with the

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Paper No. 923, University of California Citrus Experiment Station, Riverside, California.

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[3] Christie, J. R., and V. G. Perry. 1951. A root disease of plants caused by a nematode of the genus Trichodorus. Science 113: 491-493.

[4] Identified by M. W. Allen, University of California, Berkeley, as a new species, and the same species reported above by Christie and Perry. This species is being described by Allen in "A review of the nematode genus Trichodorus with descriptions of ten new species," Nematologia, January, 1957 (In press).

[5] Christie, J. R., and V. G. Perry. 1951. Removing nematodes from soil. Helminthol. Soc. Wash. Proc. 18: 106-108.

TABLE 1.—*Dry weight of foliage of alfalfa plants (var. California Common) grown in the greenhouse in soil infested with the stubby-root nematode*

Soil	Dry weight per plant (g) First cutting	Second cutting	Number of nematodes per pot at end of test
Test No. 1[a]			
Noninfested	1.8	1.6	0
Trichodorus-infested	1.2	1.1	9932
LSD at 1 per cent	0.36	0.37	
Test No. 2[b]			
Noninfested	1.8	1.4	0
Trichodorus-infested	0.2	0.2	7952
LSD at 1 per cent	0.39	0.54	

[a] Ten replicates; 7 plants per pot. At the beginning of the test, 7500 nematodes were introduced into the soil of each pot designated as infested.

[b] Ten replicates; 2 plants per pot. At the beginning of the test, 5100 nematodes were introduced into the soil of each pot designated as infested.

reduced growth of the alfalfa in infested soil. Leaves were somewhat smaller than normal on plants in infested soil but retained a normal green color. After each cutting, there were fewer new shoots or branches on plants in infested soil, and these branches did not grow as rapidly as those on plants in noninfested soil.

Roots of alfalfa plants growing in infested soil were severely stunted. The root system showed a characteristic lack of fine feeder roots (Fig. 1, B). The arrested development of lateral roots illustrated in Figure 1, C appears to result from nematodes feeding at root tips.

Role of fungi associated with stunted roots.—Since the nematode suspensions used for inoculum were not sterile, the possibility existed that fungi were the cause of the injury to the alfalfa. A number of isolations were therefore made from the alfalfa roots to find out whether pathogenic fungi were present. Parts of the root systems of plants grown in infested and in noninfested soils were washed in running tap water for 16 hours, rinsed in sterile water, and plated on water agar. Fungi developing on water agar were then transferred to potato-dextrose-agar slants and identified.

Essentially the same fungi occurred on the roots of plants growing on Trichodorus-infested and on noninfested soil. Fungi isolated were the following: *Rhizoctonia solani* Kühn, *Pythium* spp., *Fusarium oxysporum* (Schlect.) Snyd. & Hans., *Alternaria* sp., *Trichoderma lignorum* (Tode) Harz, and *Arthrobotrys oligospora* Fresen. Although several of the fungi isolated are possible parasites on alfalfa, none of them were consistently isolated from the plants grown in Trichodorus-infested soil.

A further check on the role of fungi associated with roots of the alfalfa plants was made. Equal portions of washed roots from infested and noninfested soils were comminuted in a Waring Blendor and added to steam-pasteurized soil. Alfalfa plants (var. California Common) grown in soil mixed with chopped roots of stunted plants did not differ significantly in foliage or root development after 2 months from comparable plants grown in soil mixed with chopped roots of healthy plants.

After nematode counts had been completed in nematode Test No. 1, the soil washings remaining in the Baermann funnels were used to inoculate alfalfa. The washings from both the nematode-infested and the noninfested soil were repeatedly passed through a 325-mesh screen to remove all nematodes. This nematode-free suspension was then added to sterilized soil, and alfalfa was planted. Alfalfa plants growing in soil to which the nematode-free suspension from either Trichodorus-infested soil or from noninfested soil was added showed no injury.

The results of the above tests indicate that fungi associated with the roots of alfalfa were not responsi-

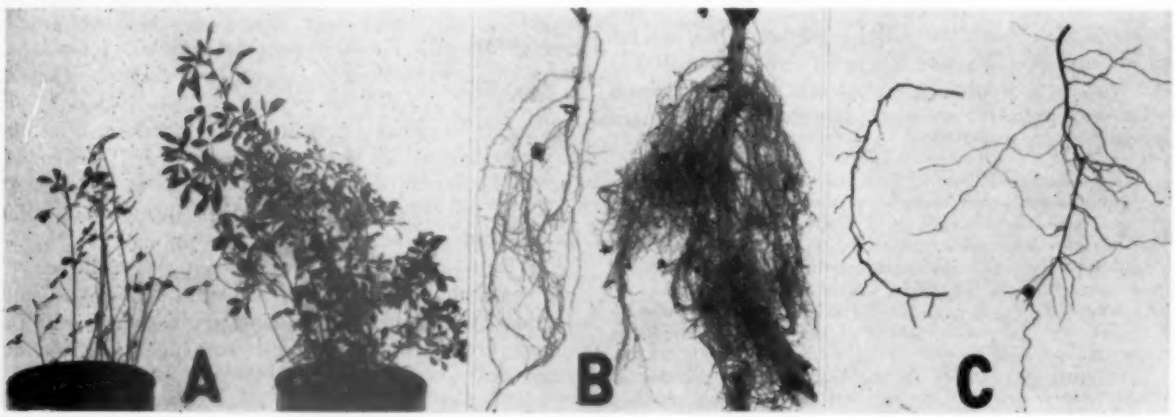

FIG. 1. A) Foliage and B) root development of alfalfa plants (var. California Common) grown in Trichodorus-infested soil (left) and in noninfested soil (right). C) Portions of lateral roots, showing typical stunting of feeder roots in infested soil (left) and normal development in noninfested soil (right).

ble for the severe stunting of the plants grown in Trichodorus-infested soil.

DISCUSSION.—The results obtained indicate that the damage observed in certain alfalfa fields may be due to the stubby-root nematode. Field populations of this nematode observed to date have generally been lower than the Trichodorus populations used in the tests reported herein. As pointed out by Christie,[6] however, field populations of *Trichodorus* sp. as determined by soil samples taken from around the roots of affected plants, may not be a reliable indication of the amount of damage that could occur. Furthermore, since injury caused by this nematode is shown primarily by reduction of foliage production without specific foliar symptoms, the damage easily may be overlooked.

The increased injury to alfalfa plants in nematode Test No. 2 probably was due to 2 factors: 1) the increased ratio of nematodes to plants (1075: 1 in the first test and 2550: 1 in the second) and 2) the warmer temperatures occurring while the test was being made.

Attempts were made to eliminate other possible plant pathogens as causal agents in these tests. The occurrence of the same fungi on the roots of stunted and healthy plants and the inability of the fungi associated with injured roots to cause a reduction in the growth of alfalfa indicate that the stubby-root nematode was the primary pathogen; however, until techniques can be developed either to free the nematodes from associated microorganisms or to grow them aseptically, it will be necessary to take into consideration the possible presence of other agents. This is particularly important in the light of recent findings made by other workers on the interaction between fungi, bacteria, and nematodes in disease production.

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[6] Christie, J. R. 1952. Some new nematode species of critical importance to Florida growers. Soil Sci. Soc. Fla. Proc. 12: 30-39.

OBSERVATIONS ON THE LONGEVITY WITHOUT FOOD OF THE BURROWING NEMATODE [1]

Wray Birchfield

SUMMARY

The burrowing nematode, *Radopholus similis* (Cobb) Thorne, was not recovered from infested soil and roots after 4 months when placed 3 ft. under the ground surface or in temperature tanks maintained at 74–76°F. It survived about 66 days at room temperature on water agar and in water.

The burrowing nematode, *Radopholus similis* (Cobb) Thorne, was reported as the causal agent of spreading decline of citrus in 1953 (3). Shortly thereafter, the State Plant Board of Florida adopted the "pull and treat" program as advocated by Suit et al (4, 5) to prevent or reduce spread of this pest. This program consists of pulling and burning infected trees within an infested area plus 2 rows of healthy trees on the edge of such areas. The cleared land is fumigated with D-D (1, 3-dichloropropene; 1, 2-dichloropropane). It may then be left fallow or planted to crops not susceptible to the burrowing nematode for 2 years before citrus is replanted. The purpose of this waiting period is to allow time for the surviving burrowing nematodes to die of starvation. It has been suggested by DuCharme (2) that they could survive only 2–3 months in the absence of a food supply, but no critical studies of the subject have been reported. The present experiments were designed to obtain some definite information on survival of burrowing nematodes in the absence of host plants.

LONGEVITY AT ROOM TEMPERATURE.—Ten burrowing nematodes, selected at random without regard to age, development, or sex, were added to each of 12 petri dishes, 8 of which contained 15 ml of water agar and 4 of which contained 15 ml of sterile water. Four of the agar plates contained a small amount of India ink to facilitate observations. All were stored at room temperatures and observed at weekly intervals. Criteria used to determine life were 1) movement, 2) coalescing of oil droplets in the intestine, 3) reaction of intestinal wall to dissection, 4) color, and 5) softness and resilience of the body wall.

The nematodes continued to move on the agar for about 4 weeks, after which they assumed a coiled, quiescent condition. A few continued to move in water for about 7 weeks. Food reserves, as denoted by oil globules in the intestinal walls, were practically exhausted after about 4 weeks when nematodes were left on either the water agar or in water. A few specimens were alive in both media on the 52nd day but apparently were dead on the 66th day.

LONGEVITY OF THE BURROWING NEMATODE IN TEMPERATURE TANKS.—Thirty-six 5-quart containers were filled with infested soil and roots and placed in temperature tanks maintained at 74–76°F. Six of these were planted with rough lemon seedlings, *Citrus limon*

[1] Accepted for publication September 16, 1956.

TABLE 1.—*Number of burrowing nematodes recovered from infested soil held fallow in temperature tank for various periods and then planted with rough lemon seedlings*

Period soil was held in tank before planting (months)	Number of test plants infested[a]	Nemas recovered
0	6/6	30
1	5/6	26
2	0/6	0
3	1/6	1
4	0/6	0
5	0/6	0

[a] Numerator is the number of test plants found to be infested, and the denominator is the number of plants examined.

Burm., at the time of establishment and 6 at monthly intervals thereafter. The last series, therefore, remained fallow 5 months before planting. The citrus seedlings and soil were watered sparingly so that free drainage, which could result in leaching of nematodes, did not occur. Weeds, as a possible source of food, were carefully removed by hand as soon as they appeared. Roots of the citrus were clipped and examined after 4–6 weeks. A final check was made of each series after about 3 months.

Burrowing nematodes were recovered in appreciable numbers only where soil had been fallow for 1 month or less before planting (Table 1). These nematodes all were active. This indicates that populations of this organism rapidly diminish and die in the absence of a host plant, even where other environmental conditions, such as temperature and moisture, are favorable.

LONGEVITY OF THE BURROWING NEMATODE IN INFESTED SOIL AND CITRUS ROOTS PLACED 3 FT. BELOW THE SOIL SURFACE.—Sixty 1-gallon cans plus 4 five-gallon crocks were filled with infested roots and soil. The roots ranged in size from the smallest feeders to about 1 in. in diameter. This material was placed in a trench 3 ft. deep and then covered with earth. The test was established July 12, and examinations for nematodes were made at monthly intervals for 6 months thereafter. Root samples were processed according to the method described by Young (6), whereas the Baermann-funnel technique as modified by Christie and Perry (1) was used for the soil.

After the first month, 4 cans of the soil and roots were examined. Five burrowing nematodes, 4 males and 1 female, were recovered alive. One male was very active. Many species of *Rotylenchus* and many typical soil forms, especially members of the Dorylaimidae, were obtained. More samples were immediately processed until a total of 20 cans of the soil and roots were processed; no burrowing nematodes were recovered.

On September 19, 6 samples were taken from 2 of the 5-gallon crocks. One of these root samples contained several healthy female burrowing nematodes. This was about 9 weeks after the start of the experiment. Only 1 female was recovered from the large crocks processed at the end of 3 months. A small amount of oil globulation remained in the intestinal walls, an indication of starvation. Thorough examination of the remaining material after 4, 5, and 6 months failed to recover any burrowing nematodes. Six 5-quart containers were filled with soil and roots of the material left from examination. These were placed in a temperature tank and planted with rough lemon seedlings. None of these seedlings became infected.

Populations of *Mononchus* spp., *Tylenchus* sp., *Aphelenchus avenae*, and members of the Dorylaiminae greatly increased during the test.

Citrus roots ½ in. or more in diameter remained alive when buried 3 ft. in the ground or placed in temperature tanks under the conditions outlined above. The larger roots formed sprouts before they formed new roots but this occurred only after 4–5 months. Small feeder roots, to which the burrowing nematode apparently is restricted in its feeding habits, died the first month under both conditions. It is believed that the burrowing nematodes starved or were otherwise killed before new roots were initiated.

DISCUSSION.—The inability of the burrowing nematode to survive more than 4 months without food is an important factor in the success of the "pull and treat" method now employed in the control program. Presumably those surviving the D-D treatment will soon die in the absence of an available food supply. The 2-year fallow that has been suggested also appears justified. The present results suggest that a shorter period might be effective; however, in practice the period probably cannot be shortened in most cases, because of the periodic inspections of the margins that must be made.

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RELATIONS BETWEEN GROWTH AND RESPIRATORY METABOLISM IN SAFFLOWER INFECTED BY PUCCINIA CARTHAMI[1]

J. M. Daly and R. M. Sayre[2]

SUMMARY

Infection of safflower hypocotyl by *Puccinia carthami* Cda. induced changes in respiration similar to those previously described for infections by other obligate parasites. The rate of respiratory activity was enhanced approximately 2-fold; the increase was correlated with increases in length and weight of the invaded tissue. During vegetative development of the parasite, the host tissue elongated approximately twice as rapidly as did healthy hypocotyls and reached a greater absolute length and weight. During sporulation, elongation ceased and increase in weight occurred more slowly.

A gradual diminution of the Pasteur effect accompanied these changes during development of the parasite. With the onset of sporulation, the ratio of anaerobic to aerobic carbon dioxide production fell to values below the expected value of 0.33; during mycelial growth of the parasite, however, the ratios were above this value although lower than those of healthy control tissue.

The data are indecisive as to whether host or parasite respiratory enzymes are responsible for the increased rates. High levels of activity were associated with pustules but not with noninfected tissue of an inoculated plant. Noninfected host tissue, however, was stimulated by 2,4-dinitrophenol to rates comparable to those that result from infection.

Increased host growth as a consequence of infection appears to eliminate "uncoupling" toxins as factors in increasing respiratory activity. The data suggest that respiratory increases arise secondarily as a result of demands for energy initially triggered by a disturbance of metabolic growth processes of the host. The inhibition of the Pasteur effect in diseased tissue occurs by 2 mechanisms: 1) increased aerobic carbon dioxide production without change in rate of anaerobic production during vegetative growth of the parasite and 2) a change in the nature of the respiratory system during sporulation.

Allen, in his excellent summary (1) of the effects of pathogens on respiratory activity of invaded tissues, has emphasized the high rates that have been found, the apparently large contribution of the host systems to the increase, and the possible role of toxins in bringing about the greater activity of the host (3). The role of the host enzymes in mediating the extra respiratory metabolism is demonstrated most clearly with the powdery mildews (3, 10), and Yarwood (14) has presented evidence that a similar phenomenon may occur in bean rust. The limited data available from the literature also indicate that in such infections the Pasteur effect is inhibited, and it was suggested that "uncoupling" agents, similar to 2, 4-dinitrophenol in action, were responsible for both the increased respiratory metabolism and the inhibition of the Pasteur effect.

Although there is no general agreement on a precise definition of the Pasteur effect, plant tissues are considered to show the effect when it is demonstrated that the rates of anaerobic glycolytic fermentation of glucose are greater than the rates of aerobic glycolysis. One widely held proposal for the mechanism of the effect postulates that rates of phosphorylation control the relative magnitudes of glycolysis and respiration aerobically (7, 8, 9) and that certain compounds "uncouple" the phosphorylation reactions from sugar breakdown, thus permitting higher rates of both processes under aerobic conditions.

Although Allen's view was modified somewhat in a subsequent article (2), several authors in recent months have subscribed to the toxic origin of the respiratory changes in disease tissue (5, 10).

In the present studies, the question of whether or not stimulatory materials are present in tissue attacked by obligate parasites was investigated. Safflower hypocotyls infected with *Puccinia carthami* Cda. were used, since the hypocotyl is a succulent and easily extractable tissue from which respiratory enzymes can be isolated for study *in vitro*. During these studies, certain observations suggested that in this instance other mechanisms account for the respiratory increases.

MATERIALS AND METHODS.— Seeds of the susceptible safflower (*Carthamus tinctorious* L.) variety N-8 were germinated in rag dolls for 24 hours. They were then dusted with a talc-teliospore mixture and transplanted to sand. Flats of inoculated and noninoculated seedlings were alternated on the floor of a greenhouse in which temperature was maintained at 15°–18° C, a range suitable for good infection on the hypocotyl.

Approximately equal numbers of plants were sampled randomly from each flat, usually in the morning. They were rinsed in running tap water to remove debris, then the hypocotyls were removed by cutting at the clearly demarked transition between primary root and hypocotyl and at the cotyledonary split in the hypocotyl epidermis. The data to be presented were obtained on samples of more than 50 and usually more than 100 plants.

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[2] Associate Plant Pathologist and Research Assistant in Plant Pathology, respectively.

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Lengths were measured to the nearest millimeter and the mean and standard error calculated from coded data. Fresh weights were obtained on 2, usually 3, samples of 15–25 hypocotyls after they were blotted on filter paper and air-dried for approximately 20 minutes. The same samples were kept at 100° C overnight for dry-weight determinations. Samples did not deviate more than 10 per cent among replicates.

Standard Warburg manometry was employed in respiratory measurements at 18° C. Aerobic carbon dioxide was measured by Warburg's direct method and anaerobic carbon dioxide production in nitrogen of 99.9 per cent purity, which did not support oxygen uptake. The gas was flushed through the vessels for 15 minutes, and then the vessels were equilibrated for 1/2 hour. Transient outbursts of CO_2 were noted during the first 15 minutes but were not measured, since only the steady-state rates of fermentation are of interest. Hypocotyl sections of approximately 2–3 mm were weighed for each vessel; the amount varied from 300 to 500 mg, but in most of the experiments reported the latter amount was used. The suspending fluid was 0.1 M KH_2PO_4. In the reported data, Q_{O_2}, Q_{CO_2}, and $Q_{CO_2}^{N_2}$ have their usual meaning. $Q_{O_2}(H)$ refers to microliters of oxygen uptake per whole hypocotyl per hour.

It should be pointed out that the length, fresh weight, and dry weight of diseased hypocotyls at any time after elongation had ceased are minimum values for these quantities. Heavily infected hypocotyls show pronounced curling and bending, and as infection progressed the tissue could not be straightened completely for length measurements. With the onset of sporulation, there was loss of dry weight through removal of spores by natural dissemination and through the handling procedures.

It was not possible to obtain equivalent amounts of infection in each of the inoculations required for the several experiments reported. Although infection was moderate to heavy on the individual plants, it was impossible to ascertain the percentage of hypocotyl tissue actually parasitized. The percentage of plants infected varied between 95 and 100 per cent in the individual samples. It is obvious that such variables influenced the magnitude of the observed changes; in addition, light intensity and day length were not controlled. These factors prevent a direct quantitative comparison among experiments; however, as will be evident, the same general relationships among respiration, growth, and parasite development were found in all experiments.

RESULTS.—Preliminary experiments established that the respiratory rates of isolated sections of the hypocotyl with visible evidence of mycelium were usually twice that of sections of noninfected plants. Occasionally, despite good vegetative development of the parasite mycelium as evidenced by the occurrence of large whitish areas on the hypocotyl, there were only slight differences in respiratory rates calculated on a fresh-weight basis. In addition, infected hypocotyls appeared larger than normal hypocotyls, and the differences in length (Table 1) were found to be statistically significant. It was noted also that unilateral infection resulted in a curvature away from the site of infection in a manner reminiscent of growth curvatures induced by unilateral application of growth hormones.

TABLE 1.—*Lengths and fresh weights of healthy safflower hypocotyls (H) and of hypocotyls infected by Puccinia carthami (D)*

Exp.	Days after planting	Fresh weight[a] (mg)		Length[a] (cm)		
		H	D	H	D	*t* Value[b]
A	18	93	119	2.69±0.08	3.09±0.07	3.77**
	24	96	134	2.94±0.07	3.26±0.07	3.24**
B	19	57	100	2.83±0.07	3.25±0.10	3.44**
	21	67	102	2.97±0.08	3.27±0.09	2.50*

[a] Samples of 50 hypocotyls.
[b] Single and double asterisks indicate significant differences in length at the 5 and 1 per cent levels, respectively.

These observations suggested that growth of the host-parasite complex, rather than "uncoupling" toxins, might be responsible for the increased rates of respiration.

Parasitic development and host growth.—The velocity and total amount of growth of both control and infected tissue was different in each of the several experiments, but the same general effects of infection were apparent in all. To illustrate these effects, 1 experiment in which growth was slow and another in which it was rapid will be discussed.

Growth in hypocotyl tissue is a consequence of elongation accompanied by water uptake and an increase in dry matter. The last may result from synthesis of active protoplasm or from the deposition of inert material such as cell walls; in the present work, no attempt was made to differentiate between these possibilities. Figure 1 illustrates the changes found with a moderately heavy infection during winter months when growth was slow.

In this series of measurements, visible symptoms of rust were not found until the 15th day when a few whitish internal areas, less than 0.5 mm in length, appeared on approximately 25 per cent of the measured sample. On the 17th day, 93 per cent of the sample showed vegetative mycelium, and by the 21st day the number and size of the infected areas was maximum (approximately 5 mm). During this time the elongation rate of the infected tissue was 0.22 cm per day whereas that of the healthy was only 0.10 cm per day. Elongation was closely paralleled by gains in fresh weight. Although no mycelium was evident on the 12th day, a slight increase in length in the diseased tissue was found, and a marked difference in fresh weight and in length became apparent on the first day that signs of the pathogen were observed. The reality of the differences at this time is substantiated by the consistent differences in dry

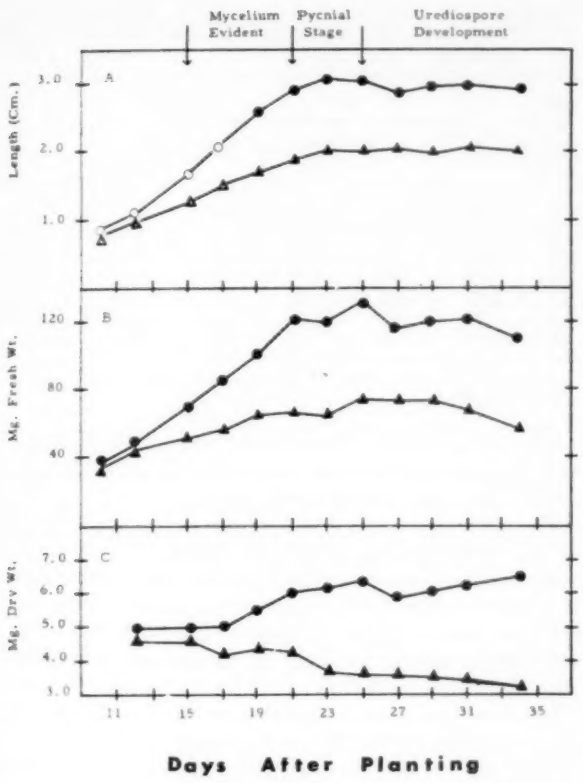

FIG. 1. Growth of healthy safflower hypocotyls (△, ▲) and of hypocotyls infected by Puccinia carthami (○, ●). A) Change in length. Open symbols indicate differences that are not statistically significant at the 1 per cent level, and solid symbols indicate differences that are significant. B) Changes in fresh weight, expressed as mg actual weight per hypocotyl. C) Changes in dry weight, expressed as mg actual weight per hypocotyl.

weight (Fig. 1, C) from the 12th to 17th day. The diseased tissue increased markedly in dry matter from the latter day until the 21st day, whereas the healthy tissue declined in weight.

By the 21st day, elongation and increase in fresh weight in both diseased and healthy hypocotyls had essentially ceased, and the increase in dry weight of the diseased tissue had slowed markedly. At this time, pycnial formation commenced, as indicated by a pinkish-brown color of infected areas; the color resulted from the production of exudate. The process continued until the 25th day when a few urediospore-bearing pustules were noted. Urediospore formation increased until the last day data were taken. A slow increase in total dry weight of the diseased tissue was associated with the onset of sporulation whereas healthy tissue underwent a continuous decline.

During this time in the development of the hypocotyl, the error involved in random sampling became clearly evident. For example, on the 27th day, the lengths and the fresh and dry weights had dropped simultaneously, presumably because the size of the hypocotyls selected on this day did not represent the true mean of the population. In order to reduce fluctuations due to sampling error, the fresh and dry weights per cm of hypocotyl were calculated (Table 2). In addition to permitting a more certain comparison of small changes in weight from day to day, this procedure reflects more precisely the interrelations between growth and dry weight increases. In both healthy and diseased tissue, dilution of cellular components as a consequence of water uptake and elongation occurred from the 12th to the 21st day, as indicated by the decline in the numerical values of the dry weight per cm. The decline was more rapid but ceased earlier in diseased than in healthy tissue as a consequence of a gain in total dry weight (Fig. 1, Bottom). Until the time of urediospore formation, the weight per unit length remained constant in diseased tissue, then increased slowly, and finally reached a value representing an increase of approximately 10 per cent over its minimum value on the 23rd day. The values for fresh weight remained nearly constant for both healthy and rusted tissue. It should be emphasized that the observed dry weights are minimum values because of the loss of spores of high density during collection and preparation of tissue.

TABLE 2.—*Fresh and dry weights of healthy safflower hypocotyls and of hypocotyls infected by Puccinia carthami*

Days after planting	Mg/cm length of healthy (H) and diseased (D) hypocotyls			
	Fresh weight		Dry weight	
	H	D	H	D
12	45.3	46.8	0.455	0.485
15	40.5	43.8	0.359	0.303
17	38.1	40.6	0.274	0.236
19	38.1	39.5	0.255	0.209
21	36.9	42.5	0.222	0.211
23	33.1	39.4	0.187	0.198
25	35.1	43.0	0.175	0.199
27	35.3	39.8	0.177	0.201
29	35.9	41.0	0.177	0.204
31	32.7	41.6	0.163	0.208
34	27.5	38.4	0.154	0.219

Nearly identical growth patterns are recorded in Table 3 for an experiment in which the growth rate of both the normal host and the infected tissue was much greater. In this instance, growth had ceased by the 17th day instead of the 21st; urediospore formation was at a maximum by the 23rd day, in contrast to the first experiment in which urediospores first appeared on the 25th day. Table 3 shows growth data in terms of length and weight per cm from which total weights per hypocotyl can be calculated.

Respiration.—With the tissues being investigated, the units used for expressing rates of respiration will influence appreciably any interpretations of the data obtained. Of the units available from our measurements, oxygen uptake calculated either for a mg of dry weight (Fig. 2, B) or for a whole hypocotyl (Fig. 2, A) appears least likely of misinterpretation. The latter

TABLE 3.—*Growth and respiration in healthy safflower hypocotyls and in hypocotyls infected by Puccinia carthami*

Days after planting	Condition of hypocotyl	Length (cm)	Fresh weight (mg/cm)	Dry weight (mg/cm)	Q_{O_2}(H)	Q_{O_2}
10[a]	Healthy	1.45 ± 0.03	29.8	2.75	6.05	1.52
	Diseased	1.53 ± 0.03	38.4	3.17	8.57	1.79
14[b]	Healthy	1.81 ± 0.04	37.1	2.36	7.99	1.86
	Diseased	2.55 ± 0.06	38.4	2.09	12.5	2.27
17[c]	Healthy	2.08 ± 0.03	37.6	1.97	7.92	1.93
	Diseased	2.71 ± 0.05	39.6	1.99	15.3	2.84
20	Healthy	2.06 ± 0.04	31.5	1.91	6.67	1.69
	Diseased	2.87 ± 0.05	39.3	2.04	18.5	3.16
24[d]	Healthy	2.11 ± 0.04	30.4	1.99	6.65	1.58
	Diseased	2.75 ± 0.05	40.8	2.37	18.3	2.78

[a] Mycelium barely evident.
[b] Visible parasite development.
[c] Pycnia present.
[d] Heavy urediospore production.

quantity reflects increasing respiratory activity as a consequence of the total activity of enzymes and substrates initially present plus those synthesized during growth, whereas the former more nearly indicates the respiratory activity for a constant unit of protoplasm, irrespective of any new synthesis of the enzymes involved.

A decline in respiratory rates in healthy tissue, which was similar to the decline in dry weights as the tissue aged, was indicated when data were expressed by either method. In diseased tissue, the rates increased rapidly during the vegetative development of the parasite and the stimulated growth of the host, reached a maximum when host growth ceased and sporulation commenced, and then appeared to decline (Fig. 2, B). The decline may have been an artifact resulting from the conversion of protoplasmic constituents and translocated materials into inactive spore materials. This would have increased the dry weight, although the activity of enzymatic units may have been the same or higher than previously. As with the healthy tissue, there was a good correlation between changes in dry weights of the hypocotyls and respiratory activity per unit of dry weight (compare with Fig. 1, B and 1, C). Similar changes in respiratory activity associated with growth were found in the tissue that developed more rapidly (Table 3).

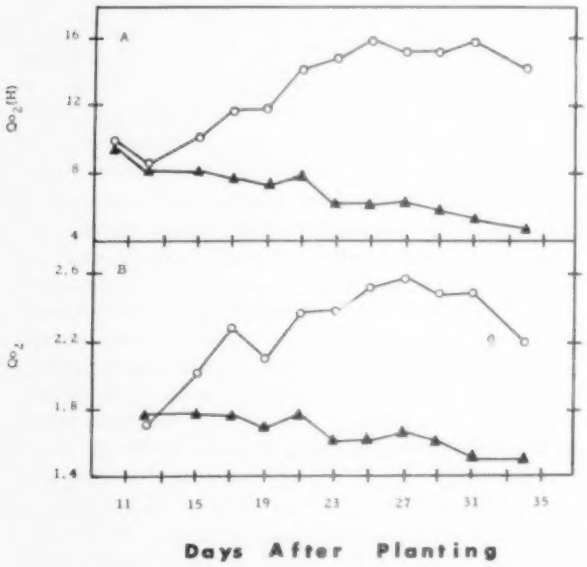

FIG. 2. Respiration of healthy safflower hypocotyls (▲) and of hypocotyls infected by *Puccinia carthami* (○). A) Microliters oxygen uptake per whole hypocotyl per hour. B) Microliters oxygen uptake per mg dry weight per hour.

TABLE 4.—*The Pasteur effect in healthy safflower hypocotyls and in hypocotyls infected by Puccinia carthami*

Age in days	Respiratory quotients in healthy (H) and diseased (D) hypocotyls					
	Q_{CO_2}		$Q_{CO_2}^{N_2}$		$Q_{CO_2}^{N_2}/Q_{CO_2}$[a]	
	H	D	H	D	H	D
10	1.42	1.59	1.22	1.23	0.86	0.77
14	1.65	2.02	1.19	1.25	0.72	0.62
17	2.01	2.95	1.30	0.95	0.65	0.32
20	1.76	3.29	1.09	0.86	0.62	0.26
24	1.76	3.00	1.11	0.75	0.64	0.25

[a] A ratio of greater than 0.33 indicates the existence of a Pasteur effect.

The Pasteur effect.—The most rigorous demonstration of the Pasteur effect is to show that glucose utilization under anaerobic conditions is greater than 1/3 the amount utilized oxidatively, but in most investigations ratios of anaerobic to aerobic carbon dioxide production larger than 0.33 are taken as indicative of the existence of the effect. The influence of age of plant on the Pasteur effect in healthy and infected safflower is shown in Table 4. The data were obtained from the same experiment reported in Table 3. The Pasteur effect was high initially but dropped with maturation of the healthy tissue. In diseased hypocotyls, the $Q_{CO_2}^{N_2}/Q_{CO_2}$ ratio was lower than in healthy hypocotyls; after sporulation it declined below the value of 0.33 expected as a theoretical minimum.

The role of host respiration in the extra oxygen uptake of diseased tissue.—It was of interest to determine whether the host tissue is capable of the high rates of respiration found during infections, since this is a basic premise of the toxin theory for the origin of the rates. 2,4-Dinitrophenol (DNP) stimulated respiration of healthy cells (Fig. 3) when applied at concentrations around 10^{-5}M, but was inhibitory at higher concentrations. The rates at 10^{-5}M were greater for healthy tissue than for diseased tissue of the same age (Fig. 3). Similar results are reported by Millerd and Scott (10).

It is not possible, however, to assume that the respiration of diseased tissue is that of the host enzymatic mechanism since we have consistently observed that the increased rates are closely associated with visibly infected areas of the hypocotyl (Table 5).

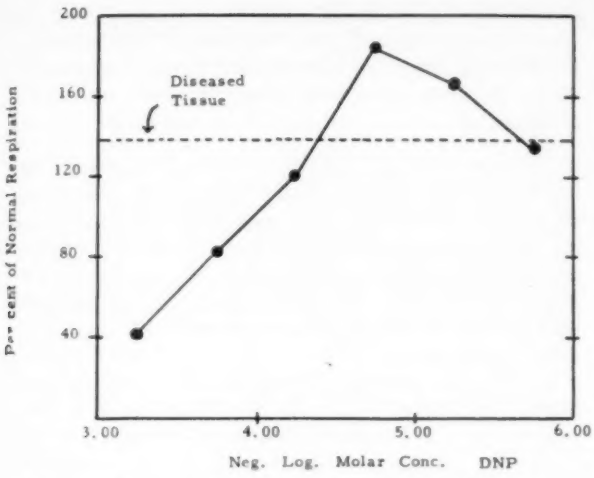

FIG. 3. The effect of 2,4-dinitrophenol (DNP) on oxygen uptake in normal hypocotyls of safflower. The dotted line shows the percentage increase in respiration caused by infection by *Puccinia carthami* on hypocotyls of the same age.

In this experiment, a heavily infected lot of hypocotyls was randomly sorted into 2 groups: 1 was sectioned into segments as usual, and in the other, the areas of the hypocotyl showing visible mycelium were separated from areas showing no signs of the pathogen. The respiration of the infected portions was slightly higher than the rates for the entire diseased hypocotyl, whereas that of the apparently healthy sections was only slightly higher than that of the noninfected hypocotyls (Table 5). The latter was not unexpected because of the strong possibility that the apparently healthy segments may have contained small amounts of mycelium not visible to the unaided eye. Since the hypocotyls were heavily infected, it would be expected that whole diseased hypocotyls would have rates of respiration only slightly lower than the individual pustules found on them.

DISCUSSION.—The hypothesis originally suggested by Allen to explain changes in respiratory rates as a consequence of infection by certain obligate parasites is an interesting model useful in designing experimental approaches to the nature of host-parasite interactions. According to this concept, the adenylic acid cycle is the rate-limiting factor in the respiration of normal host tissue presumably because the ratio of adenosinetriphosphate (ATP) to adenosinediphosphate (ADP) is high. As Meyerhof (9) and Johnson (7) have pointed out, low levels of ADP restrict the rate of respiration, since it is required as a phosphate acceptor in the generation of high-energy phosphate that is obligately linked to the enzymatic reactions of the classical Embden-Meyerhof-Parnas scheme of glycolysis and the Krebs' citric acid cycle of oxidation. The toxin theory proposed a material which "uncouples" the rate-controlling adenylic system of the host during parasitic development (at point A in Figure 4) in a manner analogous to the uncoupling action of 2,4-dinitrophenol. Tepley has presented evidence indicating that this compound increases the activity of adenosinetriphosphatase (13).

TABLE 5.—*Respiratory rates of infected and noninfected areas of safflower hypocotyl*

Type of hypocotyl tissue	Q_{O_2}	Percentage of Q_{O_2} of whole healthy hypocotyl
Whole, healthy	1.04	
Apparently healthy sections from infected tissue	1.22	117
Whole, diseased	1.81	174
Infected sections from infected tissue	1.98	190

The model would account for several aspects of respiratory changes that have been described during infection. In addition, the deleterious effects of some infections could conceivably arise from the postulated toxin since uncoupling would effectively prevent normal synthesis and maintenance of host tissue because of the lack of formation and utilization of high energy phosphate bonds. Recently, Millerd and Scott (10) and Farkas and Kiraly (5) have tended to accept this view but no positive evidence in support of it has been presented, and the circumstantial data advanced is open to serious criticism. Attempts to isolate or to demonstrate a toxin with the above characteristics, in our experience and in that of Farkas and Kiraly, have been unsuccessful.

The data obtained with safflower rust would appear to eliminate the possibility of an "uncoupling" toxin in this disease, since host growth, as well as mycelial development and sporulation, is positively correlated with the increases in respiration. The main biological effect of synthetic or of natural (8) "uncoupling" agents is the inability of cells and tissue to perform those synthetic activities of growth and maintenance requiring the expenditure of respiratory energy. In addition, the failure to obtain stimulation of respiration at a distance from the infected site would also indicate the absence of a toxin although it may be of limited diffusibility (3).

It would appear more reasonable that the changes in respiratory rates result from stimulation, as a result of infection, of some metabolic or hormonal process (Point B in Fig. 4) that in turn is geared to the adenylic cycle through a requirement for the high-energy bonds of ATP. Utilization of ATP would lower the ATP/ADP ratio, resulting in a resumption of high respiratory activity as a consequence of the demand for the necessary energy in vegetative growth of the host and parasite and in the sporulation of the parasite. It might be considered that infection of safflower hypocotyl by *P. carthami* differs from infections of foliar tissue by obligate parasites, but it is entirely possible that the same triggering mechanism

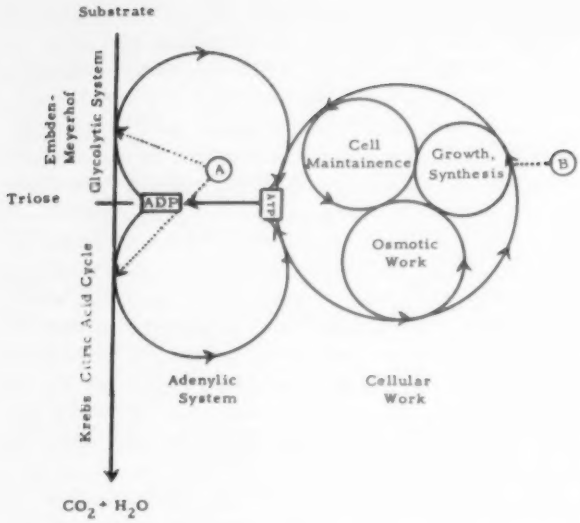

FIG. 4. Representation of relations among respiration, growth, and "uncoupling" agents. The last act essentially to dissociate the energy-producing reactions of substrate catabolism from the adenylic system (Point A), thus blocking the performance of work but causing increases in enzymatic activity of the enzymes of glycolysis and the Krebs' citric acid cycle. Respiratory stimulation as a result of increase in cellular work would correspond to action at Point B, causing increased turnover of the adenylic system.

is present in these cases and that observable host growth is not found because the host tissue has irreversibly lost the capacity for growth. Pilet (11) has reported increase in size of Sempervirens leaves infected with a rust fungus. The accumulation of phosphorus (6) and other materials (15) in infected areas of leaves, as well as a number of other physiological phenomena that occur during infection by obligate parasites, indicates high metabolic activity and a considerable requirement for energy.

The inhibition of the Pasteur effect also can be accounted for in our hypothesis. During mycelial development and host growth, the rates of anaerobic CO_2 production of both healthy and diseased tissue are approximately equal (Table 4, 10 and 14 days) and probably represent the maximum capacity of the enzymes of the fermentative system of the host. In diseased tissue during this period, the rate of aerobic CO_2 production is higher because of the requirement of energy for additional growth. Consequently the $Q_{CO_2}^{N_2}/Q_{CO_2}$ ratio is considerably smaller in diseased tissue than in healthy tissue, but no really significant difference in respiratory mechanisms can be ascribed to the numerical differences in the ratio. During sporulation, the $Q_{CO_2}^{N_2}/Q_{CO_2}$ ratio is actually below the theoretical limit of 0.33 expected if the Embden-Meyerhof pathway of anaerobic breakdown of glucose to alcohol is operating. We have obtained several lines of evidence showing that the hexose monophosphate shunt (12) is the main respiratory pathway during sporulation of the pathogen, whereas in healthy tissue the Embden-Meyerhof scheme is of major importance. Since the monophosphate shunt depends on direct oxidation of carbon 1 of glucose by molecular oxygen, its occurrence would explain the $Q_{CO_2}^{N_2}/Q_{CO_2}$ values below 0.33. This evidence will be presented more fully in another paper (4). Our present information does not permit an answer to the question of the relative participation of host or parasite enzymes, and techniques other than the ones employed in previous studies must be developed in order to obtain an answer that is not ambiguous.

The present data do demonstrate that "uncoupling" toxins cannot account for the respiratory increases but rather that a more logical area for investigation of the causes for increased metabolic rates lies in studies on the hormonal control of growth and respiration in infected tissue. Since vegetative growth of the parasite apparently shares or requires a physiological system that is involved in host growth, infection by *P. carthami* on the hypocotyl of safflower may be an extremely valuable tool for an analysis of these relationships.

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RATE AND TIMING OF FUNGICIDAL SOIL TREATMENTS[1]

W. H. Cram and O. Vaartaja

SUMMARY

Unsterilized soil was sown with seeds from trees of 3 genera and inoculated with *Rhizoctonia solani* Kühn. Four rates of weekly drenches of Orthocide 75 (75 per cent captan) and Tersan (75 per cent thiram), and various timing schedules of Tersan applications were tested. Damping-off, caused mainly by the inoculum fungus and partly by the natural Pythium flora in soil, was largely controlled by the weekly application of 0.1 g/sq. ft. of either fungicide; for certain trees the optimum rate was 0.5 g rather than 0.1 g. In general, mortality was slightly higher with the 0.5-g rate and considerably higher with the 2.5- and 10-g rates than with the lowest rate, because of phytotoxicity and crust formation on the soil. Tersan at the very high 10-g rate gave a statistically significantly better seedling stand than did the check treatment and had less adverse effect than the 10-g rate of Orthocide 75. The adverse effects of the weekly applications of fungicides appeared only after the fungicides had accumulated in the soil. Very good control without phytotoxicity was obtained with special schedules where the first application was fairly heavy (0.6 g Tersan per sq. ft.) and subsequent applications at 1- or 2-week intervals were light (0.1 g). Fungal population in the soil and the frequency with which *R. solani* was isolated from diseased seedlings were reduced, even with the low weekly rates; they approached zero with high rates. The fungicides seemed to have no consistent effect on bacteria other than to allow them to increase to very high population levels.

A common means for controlling damping-off of many plant species is to treat the seed with fungicides. This may not always be effective for species that remain susceptible for a long time, especially in an environment favoring the disease. In such cases, repeated applications of fungicides of low phytotoxicity are required. To find those fungicides that have a wide margin between *dosis curativa* and *dosis tolerata*, 109 chemicals were screened in a laboratory test (17). Thiram and captan fungicides met this requirement in the tests, in which *Pythium debaryanum* Hesse and *Rhizoctonia solani* Kühn were used as the pathogens and seed of *Pinus banksiana* Lamb. as the host.

Higher dosages of fungicides are required for soil treatments in practice than those that inhibit the pathogens in laboratory tests. The higher dosages are needed to compensate for 1) the failure of fungicides to spread uniformly throughout the soil; 2) adsorption by certain soils; 3) gradual diffusion in soil; 4) dilution after irrigation and rains; 5) chemical inactivation; and 6) biological breakdown. Because all these factors vary irregularly, no simple rigid rules can be followed in application schedules, and routine field tests help little in evaluating the importance of these factors. Thiram has successfully controlled damping-off in various agricultural and garden crops (6,8,10,11,14) and forest trees (1,5,12,13,19). Recently captan also has given promising control with cotton (3), sugar beet (9), and conifers (13,19). The situation is complicated, however, because phytotoxicity and failures in control also have been reported with these same fungicides (4,6,7,9,10,14,19,20). In general, no attention has been given to maintaining the necessary concentration of fungicides in the soil.

The present study was designed to obtain information on varying rates and intervals of application of thiram and captan. Damping-off is common and difficult to control in the tree species tested (12,13,16, 18,19). A strain of *R. solani* that had proved highly virulent to several tree species (18) was used as inoculum. *R. solani* is also known as one of the most common causes of damping-off of various species and is relatively resistant to fungicides (8, 16, 17, 19).

METHODS.—*Experiment 1.*—Seed from 2 trees each of Colorado spruce (*Picea pungens* Engelm.), Scots pine (*Pinus sylvestris* L.), and caragana (*Caragana arborescens* Lam.) were standardized by screening, surface sterilized with 0.5 per cent mercuric chloride solution, and rinsed with water. Seed of these 3 species were stratified in moist sand at 5°C for 29, 14, and 14 days, respectively, then sown in flats in a mixture of sand and alkaline loam from old seedbeds.

A culture suspension of *R. solani* (*Corticium praticola* Kotila) was sprinkled on the flats. Tersan and Orthocide 75 were applied as surface drenches at 4 rates: 0.1, 0.5, 2.5, and 10 g/sq. ft. These rates approach 10, 50, 250, and 1000 lb./acre, respectively, or 1/5, 1, 5, and 20 times respectively, those recommended by the manufacturers. Tersan contained 75 per cent thiram (bis(dimethylthiocarbamoyl)disulfide), and Orthocide 75 contained 75 per cent captan (*N*-(trichloromethylthio)-4-cycloxene-1,2-dicarboximide).

The soil medium was maintained at an available moisture content of 70–80 per cent as indicated by the Bouyoucos (2) block method; tap water was applied daily as required. Air temperature varied mainly between 10 and 30°C. In the check (no fungicides) treatment, the average germination was 75 per cent and seedling mortality 54 per cent.

A split-plot design was utilized with 3 replications. Data on speed of germination (emergence), on emerg-

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TABLE 1.—*Damping-off of 3 tree species in greenhouse flats given 9 weekly drenches with fungicides (Experiment 1)*

Fungicide and rate (g/ sq. ft.)	Relative emergence (check = 100)[a] of 2 progenies of each of indicated species							Relative stand (check = 100)[a] of 2 progenies of each of indicated species						
	Picea pungens		*Pinus sylvestris*		*Caragana arborescens*		Mean	*Piceae pungens*		*Pinus sylvestris*		*Caragana arborescens*		Mean
	155	820	493	593	V-15	V-16		155	820	493	596	V-15	V-16	
Orthocide 75														
0.1	104	149*	108	101	106	121*	114*	161	216	180*	380	105	162*	168*
0.5	129*	131*	85	108	115*	122*	114*	170	111	106	420*	118	173*	160*
2.5	112	118	114*	110*	90	125*	111*	100	74	123	320	86	161	130
10	100	90	71*	109	112	126*	102	(30)[b]	(0)[b]	(11)[b]	(65)[b]	(110)[b]	(157)[b]	(86)[b]
Tersan														
0.1	146*	110*	126*	115*	95	122*	116*	274*	275*	187*	275*	119	167*	186*
0.5	128*	111*	139*	115*	98	119*	117*	197	240*	172	286*	125	167*	176*
2.5	128*	89	114*	110*	100	111*	108*	204	205	105	232*	121	149	155*
10	132*	125*	98	111	103	120*	115*	226	155	79	225	125	150	149*

[a] Each figure is based on counts in 3 flats, in each 25 seeds of each tree. An asterisk indicates that the difference between the indicated figure and that of check is significant at 0.05 level.
[b] These data were not included in the analysis.

ence incidence, and on stand were subjected to analysis of variance to determine the significance of differences between treatments and check. Twenty-five seeds of each species were sown for each treatment in each replicate.

Attempts were made to reisolate the pathogens by surface sterilizing and plating the seedlings immediately after the disease was detected. At the conclusion of the experiment, samples of surface soil (uppermost 1/4 in.) were taken, and various dilutions were mixed with potato-dextrose agar to determine the number of bacteria. When the number of fungi and actinomycetes was to be determined, streptomycin and rose bengal (each at 30 mg/l) were added to the agar medium to suppress the otherwise predominant bacteria.

Experiment 2.—Colorado spruce and caragana were tested with Tersan only at various initial applications and subsequent drenches of 0.1 g/sq. ft. The pelleting treatment was made by shaking 1 part of an aqueous solution of 2.5 per cent methyl cellulose with 20 parts of seed and 2 parts of Tersan.

The tests were replicated twice at each of 2 slightly different soil moisture levels; however, moisture levels produced no significant effects. Otherwise, the experiment was conducted in the same way as Experiment 1. In the check (no fungicide) treatments, the average germination was 80 per cent and seedling mortality 41 per cent.

DISEASE AND PHYTOTOXICITY.—Speed of germination for all trees was greatly reduced by the heaviest applications of Orthocide 75 and Tersan in Experiment 1. The lighter applications, even 0.1 g/sq. ft., significantly reduced the speed of germination of Colorado spruce and Scots pine. In Experiment 2, with smaller total amounts of the fungicide, no statistically significant reduction in germination speed resulted from soil drenches, but the heavy pelleting reduced speed of germination of caragana seed. These reductions in speed of germination were attributed mainly to the phytotoxicity of the fungicides. Another cause may have been the impervious crust that gradually formed on the soil surface when the fungicides were used in very large amounts. This was particularly noticeable with the heaviest rate of Orthocide.

Whereas the drench with the lowest rate of Orthocide 75 greatly increased stands on the average and for many of the trees, there were no statistically significant increases with the heaviest rate (Table 1). On the contrary, the heaviest rate resulted in decreases rather than increases for some of the trees. Similarly, whereas the lowest rate of Tersan gave large significant increases for most of the trees, the heaviest rate gave only increases that were consistently smaller and not significant (except the mean). For most of the trees, the stands with the moderate rates were intermediate.

There was some interaction with stands and tree progenies (Table 1). This was caused partly by the different levels of the disease in different progenies. For instance, the high resistance and vigor of the progeny V-15 precluded any large and significant effects due to fungicides. As a whole, the data were considered to suggest that the optimum rate generally was about 0.1 g (most trees) or close to this (trees 596 and V-16). With increased rates, the harmful effects appeared to counteract the disease control, clearly so with Orthocide 75 and less obviously and very gradually so with Tersan. This is in accord with the data on speed of germination. In contrast, the emergence incidence showed almost no correlation with the rates (Table 1). During the main germination phase, the fungicides may not yet have been accumulated in amounts sufficient to prevent germination. The optimum rate for emergence of many of the trees

TABLE 2.—*Damping-off of 2 tree species in greenhouse flats given Tersan (75 per cent thiram) drenches at various schedules (Experiment 2)*

Rate of first application (g/sq.ft.)	Later applications (at 0.1 g/sq. ft.)		Relative emergence (check = 100)[a] of 2 progenies of each of indicated species					Relative stand (check = 100)[a] of 2 progenies of each of indicated species				
			Picea pungens		*Caragana arborescens*		Mean	*Picea pungens*		*Caragana arborescens*		Mean
	No.	Average interval (days)	C–12	C–27	V–2	V–15		C–12	C–27	V–2	V–15	
0.6	4	14	118*	113*	141*	118*	121**	171*	157*	311*	161*	185**
0.6	6	7	118*	111	141*	120*	121**	178*	160*	322*	163*	190**
0.6	11	3.5	92	109	134*	110	110*	109	162*	305*	138	162*
0.1	6	7	112	113*	134*	108	116*	146	157*	200*	144	157*
Seed pelleted	6	7	118*	111	85	98	104	178*	131	162	122	145*

[a] Each figure is based on counts in 4 flats, in each 25 seeds of each species. Single and double asterisks indicate that the difference between the indicated figure and that of check is significant at 0.05 and 0.01 levels, respectively.

appeared to be higher than 0.1 g, in some cases even one of the heavy rates (2.5 or 10 g, see Table 1).

This situation gave bases for 2 hypotheses. 1) For early disease control, the first application needs to be moderately heavy. 2) The later applications may be light and yet give disease control; they should not be high if the gradual accumulation of the fungicide in phytotoxic quantities is to be avoided. In accordance with this reasoning, a moderate initial rate of Tersan with 6 light subsequent weekly rates materially increased both the emergence and the final stand (Table 2). Apparently an optimum fungicidal concentration was approached and maintained. Pelleting appeared phytotoxic to caragana seed as indicated by reduced (Table 2) and slow emergence.

Both Tersan and Orthocide 75 gave high control with weekly drenches at rates of 0.1 and 0.5 g/sq. ft. (Table 1). Responses to these rates of the 2 fungicides did not differ significantly. The plants, however, tolerated very high concentrations of Tersan better than they did of Orthocide 75. Some progenies of the same tree species (e.g. *P. sylvestris*, Tables 1, 2) exhibited different degrees of tolerance to the fungicides.

Phytotoxic effects on seedling vigor were investigated. Weights of tops were determined, and root development was visually estimated for a sample of the surviving seedlings at the conclusion of Experiment 1. Fungicides appeared to inhibit the top growth of most seedlings but not to a degree that was statistically significant. The root development of conifers was abnormal under the experimental conditions of constant high moisture; many roots had root hairs, but mycorrhiza were scarce or lacking. Most conifer seedlings, however, had numerous short roots in all treatments except the highest rate of Orthocide 75. Root nodules were numerous on caragana in all treatments.

EFFECTS ON MICROFLORA.—*Soil flora.*—Table 3 shows the results of soil platings at the termination of Experiment 1. Fungicides greatly reduced the populations of fungi and actinomycetes in the surface soil. Applications of either fungicide were generally accompanied by an increase in the number of bacteria, but large deviations appeared between the replications.

Similar trends in soil flora were also demonstrated in Experiment 2; however, the reduction in number of fungi was smaller than in Experiment 1. This was explained partly by the lower application rates and partly because the dominating soil species, a species of *Penicillium*, was relatively tolerant to thiram. The following data illustrate the trend and variation in bacterial flora. The average number of bacterial colonies (in 1000 per g soil) from samples of the Tersan treatment with heavy initial application and 11 low semiweekly applications was 5250 (range 2 to 24,000), whereas it was only 150 (range 50 to 340) in the check treatment.

Reisolation.—The general results from platings of diseased seedlings from Experiment 1 also are shown in Table 3. These data suggest that in the check and in the 0.1-g weekly treatments, damping-off was commonly caused by the inoculum fungus, *R. solani*. Details of isolation data also indicated that the naturally occurring species of *Pythium* caused some mortality in the same treatments. Similarly, the isolation data from Experiment 2 suggested that the mortality was caused mainly by *R. solani* but partly by *Pythium* sp.; however, because of less comprehensive data and generally lower levels of disease and reisolation, no such consistent trends could be established as in Experiment 1 (Table 3).

The conclusion that mortality following very heavy applications of fungicides resulted primarily from chemical injury and from the effects of crust formation is supported by several facts. 1) A high percentage of the platings from the 0.5- and 2.5-g treatments yielded only bacteria or no organisms at all; the percentage incidence was still higher in the 10-g treatment. 2) Some of the seedlings considered as dying began to grow after being transferred to agar plates. 3) After the heaviest treatment, the only common isolate that was suspected of pathogenic potentials was a species of *Fusarium;* however, no conclusion could be reached concerning its virulence under the particular conditions of the experiment because this

TABLE 3.—*Number of fungi and actinomycetes in soil and incidence of Rhizoctonia solani after 9 weekly drenches with fungicides (Experiment 1)*

Fungicide and rate (g/sq. ft.)	No. of organisms in 1 g of soil[a]		Percentage of seedlings infested by *R. solani*[b]
	Fungi	Actinomycetes	
	x 10^2	x 10^4	
Orthocide 75			
0	940	13	64
0.1	150	5	17
0.5	100	5	2
2.5	59	0	0
10	7	0	0
Tersan			
0	930	15	63
0.1	105	25	12
0.5	105	5	3
2.5	0	0	2
10	0	0	0

[a] Evaluated by the dilution-plate technique. Number of bacteria not given because of large inconsistent variation. Each figure is the mean of 2 replicates.
[b] Based on platings of diseased seedlings.

fungus was also isolated from healthy seedlings.

DISCUSSION.—The results of this study show that good control of damping-off can be obtained with thiram and captan soil drenches. This is in agreement with several previous reports (1, 5, 6, 8, 10, 11, 12, 13, 14, 19). Good control was attained even with the very virulent pathogen, *R. solani*, on highly susceptible tree species germinating in the greenhouse in moist alkaline loam, an environment known to favor damping-off. In damping-off control, either a seed treatment or a single drench of a fungicide at the time of seeding is the common practice; however, Strong's (13) convincing results with susceptible conifers indicate the advantages of repeated applications. The present results suggest a means of overcoming the danger of phytotoxicity (19, 20) and still obtaining effective control. This is to give a heavy initial application and several lighter subsequent applications. Richardson (11) showed that thiram persists and acts in soil for a while but gradually declines, the rate of decline depending on the soil. In sand the active period lasted over 63 days, but in compost soil for only 4–7 days. In clay loam, thiram has controlled Aphanomyces damping-off of sugar beets for 45 days (6); it lasted longer in dry soil than in wet soil. In the moist loam used in the present study, thiram gave best results with light supplements at intervals of 1 or 2 weeks.

The gradual development of phytotoxicity of thiram and captan demonstrated in this study and in the laboratory (4, 17) suggests relatively wide margins of safety. In soil, thiram had a wider margin of safety than did captan.

The selectivity of thiram (11, 15, 17) and captan (17) was also evident in this study. Both fungicides were more toxic to the pathogens (*R. solani* and *Pythium* sp.) than to the saprophytes (species of *Penicillium* and *Fusarium*), and both allowed and apparently encouraged the growth of bacteria. This selectivity may exert an indirect biological control against the pathogens.

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FACTORS AFFECTING SYMPTOM EXPRESSION IN FUSARIUM WILT OF DIANTHUS[1]

John R. Hood and Robert N. Stewart[2]

SUMMARY

Root-dip and stem-wound inoculations with *Fusarium oxysporum* f. *dianthi* induced symptoms of carnation Fusarium wilt more quickly than did inoculations by infestation of the soil. These were also the most efficient methods for mass inoculations. No significant amount of cross infection occurred when pots of inoculated plants touched pots of noninoculated plants. Approximately 1500 fungus bodies per ml of inoculum were needed to induce symptoms when inoculation was by the root-dipping method.

The interval between inoculation and appearance of symptom was progressively decreased by increases in air or soil temperatures between 15° and 32°C. The results indicate that the best combination for the routine testing of carnations for susceptibility to Fusarium wilt is a soil temperature of 26° and an air temperature of 21° or 26°C.

Existence of 3 pathogenic races of *F. oxysporum* f. *dianthi* was established by tests on 11 commercial varieties of carnations.

Fusarium wilt, caused by *Fusarium oxysporum* f. *dianthi* (Pril. & Del.) Snyd. & Hans. has long been a serious disease of carnations (*Dianthus caryophyllus* L.) in greenhouse plantings. Bickerton (2) reviewed the relevant literature and described many of the important characteristics of the disease, but further information is necessary for breeding carnations resistant to Fusarium wilt. Selection of resistant individuals requires knowledge about the various factors influencing symptom expression by the host. Therefore, inoculation technique, inoculum density, environmental conditions, and differential host susceptibility were investigated.

MATERIALS AND METHODS.—Cultures were obtained from single spores of Fusarium isolates from diseased carnation plants collected in several states. The cultures of these geographical isolates were designated by the following numbers: 5, from Colorado; 9, from California; 11, from New York; 12, from New York; and 14, from Connecticut. All inocula used were prepared from mass-transfer subcultures of the original cultures derived from single spores. They were grown on modified Tochinai agar prepared by dissolving 0.25 g of magnesium sulfate, 0.5 g of monopotassium phosphate, 10 g of Difco peptone, 20 g of sucrose, and 20 g of Difco agar in 1 liter of distilled water.

Standard inoculum was prepared by transfer of the pathogen to 30 ml of modified Tochinai broth in 125-ml Erlenmeyer flasks. The cultures were held for 2 weeks at 26° ± 1°C, ground with a Waring Blendor in 100 ml of tap water for ½ minute at slow speed and ½ minute at high speed, and diluted to 250 ml with tap water. The inoculum contained broken hyphae, macrospores, and microspores; subsequently, these are referred to as fungus units.

Cuttings of clonal varieties of carnations rooted in sand were used in all experiments except the host-range studies. Seeds for the host-range tests were obtained from domestic commercial sources.

One method of inoculation consisted of dipping the roots in tap water to remove the sand, immersing the roots in standard inoculum for 1 or 2 minutes, and then potting the cuttings in sterilized soil in 2-in. pots. Another method consisted of inserting a bit of fungus growth on agar into knife cuts on the lower stem. In a third method, cuttings were potted in sterilized soil and inoculated after 2 weeks by pouring 10 ml of standard inoculum around each plant after its roots had been wounded by insertion of a 6-in. pot label into the soil near the stem. Fusarium grown on grain was used as inoculum in a fourth method; 8 to 10 infested kernels were placed in contact with the roots as rooted carnation cuttings were potted.

Potted carnations were sunk in sand, rim to rim, on greenhouse benches, and the soil temperature was maintained at approximately 26°C by electric heating

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FIG. 1. Carnation cuttings (variety Aura) inoculated with isolates 5, 11, 12, and 14 of *Fusarium oxysporum* f. *dianthi*, showing unilateral symptoms typical of Fusarium wilt. The control plant on the right had dark-green foliage in contrast to the lighter foliage of diseased plants.

cables buried in the sand. Air temperature was maintained at about 18° at night and 21°C during the day.

All plants were examined 3 times each week for wilt symptoms; plants wilting and dying from any cause were recorded as expressing symptoms. The appearance of typical Fusarium wilt symptoms in most wilting and dying plants indicated that they were infected with *F. oxysporum* f. *dianthi*. For purposes of statistical analysis, all plants remaining healthy or unwilted at the end of the experiment were arbitrarily recorded as showing symptoms on the terminal date. Such a terminal value for each plant in the experiments is necessary to permit statistical treatment of the data, and the differences in treatment means that existed at the penultimate reading are preserved. Because of this method of recording the data, the number of days between inoculations and the appearance of symptoms (or the terminal date) is referred to herein as the "days-to-symptoms index." All data on the days-to-symptoms index were analysed statistically; in all cases references to significant differences mean statistically significant differences.

EXPERIMENTAL RESULTS.—The fungus isolates used in these experiments were identified as *F. oxysporum* f. *dianthi* by comparison of their morphological and cultural characteristics with published descriptions of this form; however, there were variations in pigmentation and spore size within and among the isolates. The identity of each isolate was further established by the fact that it produced typical unilateral symptoms of Fusarium wilt on inoculated carnation plants (Fig. 1). When the inoculum density was high and the inoculated cuttings were kept at high soil and air temperatures, many showed a general multilateral discoloration and wilting that resulted in relatively early death from Fusarium wilt.

In a preliminary experiment, rooted cuttings of the carnation variety Aura were inoculated by dipping the roots in standard inoculum of isolate 5 of Fusarium, and 10 cuttings for each of 3 guard rows, plus 10 cuttings for a check row, were dipped in water. When the plants were potted, the pots were sunk in sand in rows with adjacent pots rim to rim. Single guard rows were placed between adjacent treatments and a double row between replications. The test was replicated 3 times in randomized design, and observations were made for 80 days, March 23 through June 11, 1954. Of the 30 fungus-inoculated plants, 83 per cent developed irreversible wilt symptoms, whereas only 0.8 per cent of the 120 noninoculated ones, including those in the guard rows, showed similar response. Since no significant amount of cross infection by the pathogen was observed, the plants in all subsequent experiments were spaced as described with the guard rows omitted.

Inoculation technique.—Aura carnations were inocu-

TABLE 1.—*Days-to-symptoms indexes*[a] *for carnations (variety Aura) inoculated by 6 methods with isolate 5 of Fusarium oxysporum f. dianthi*

Method of inoculation	Mean days-to-symptoms index[a]	
	Sterile "inoculum"[b]	Fusarium inoculum
Dipping roots in standard inoculum	44.0	11.0
Inserting agar culture into stem wound	42.1	15.0
Adding grain inoculum to the potting soil	45.9	26.2
Potting in sterilized soil infested with Fusarium 2 weeks prior to potting	47.7	30.2
Potting in unsterilized soil infested with Fusarium 2 weeks prior to potting	43.9	39.7
Pouring standard inoculum on soil around cuttings potted 2 weeks previously	49.0	39.1
LSD (0.05)	4.9	
LSD (0.01)	6.6	

[a] Mean number of days from inoculation to the appearance of symptoms or to the termination of the experiment (49 days). Each figure is the mean for 40 plants.

[b] Control plants were treated like the inoculated ones except that the Fusarium inoculum was omitted.

lated with isolate 5 of *F. oxysporum* f. *dianthi* by 6 different methods (Table 1). Dipping of roots into standard inoculum or insertion of fungus growth on agar into stem wounds resulted in earlier appearance of symptoms than did any of the other methods tested, as shown by the highly significant differences among the mean indexes. It is of interest that plants potted in infested soil that previously had been steam sterilized developed symptoms in a significantly shorter period of time than did those potted in infested soil that had not been sterilized. In an experiment mentioned below, no significant difference was found between the days-to-symptoms index for plants inoculated with standard inoculum and that for those inoculated with microconidial inoculum.

Inoculum density.—Standard inoculum and 6 dilutions were applied to cuttings by the root-dip method, and a water check treatment was included. Treatment units consisted of 10 plants each, arranged in a randomized-block design replicated 3 times. Symptoms developed as soon when a 1:10 dilution of standard inoculum was used as when it was used undiluted (Table 2). Symptom development was delayed when the inoculum was diluted 1:100 and further still when it was diluted 1:1000. Four dilution plates were made from each dilution of the standard inoculum, and colonies were counted after 24 hours of incubation at about 26°C. The over-all mean showed that standard inoculum contained approximately 152,000 living fungus units per ml. The mean days-to-symptoms indexes for plants inoculated with inocula containing less than 1500 fungus units per ml did not differ significantly from that for plants treated with water.

TABLE 2.—*Days-to-symptoms indexes for carnations (variety Aura) inoculated with standard inoculum*[a] *of isolate 5 of Fusarium oxysporum f. dianthi at various dilutions*

Dilution	Mean days-to-symptoms index[b]
None	12.8
1–10	15.0
1–100	21.8
1–1000	43.8
1–10000	43.9
1–100000	43.3
1–1000000	45.5
Water (check)	47.4
LSD (0.05)	4.6
LSD (0.01)	6.6

[a] Standard inoculum was prepared by growing a culture of *F. oxysporum* f. *dianthi* for 2 weeks at 26°C on modified Tochinai broth, grinding for 1 minute in a Waring Blendor, and diluting to 250 ml with tap water.

[b] Mean number of days from inoculation to the appearance of symptoms or to the termination of the experiment (49 days). Each figure is the mean for 30 plants.

Environmental conditions.—A factorial experiment in randomized-block design was made to test the effect of several treatments on symptom expression in the carnation variety Aura inoculated with Fusarium isolate 5. The treatments were 1) watering once a day versus watering twice daily; 2) use of standard inoculum versus use of microconidial inoculum from a shake culture versus use of water only; and 3) wounding of roots by crushing them with pliers versus no wounding other than that incurred during transplanting. As stated earlier, results with standard inoculum were not significantly different from those with microconidial inoculum, but there was a highly significant difference between those with fungus inoculum and those with water. Plants inoculated with standard inoculum and watered twice daily developed symptoms sooner than did those watered once a day; the mean indexes for the 2 sets of plants differed significantly. Crushing the roots of cuttings delayed symptom expression regardless of watering treatments. In another factorial experiment in randomized design, there were no significant differences or interactions in treatments involving 1) application of 0.1 per cent indolebutyric acid, 2) standard potting soil versus a 1:1 mixture of soil and sand, and 3) standard soil versus standard soil with nutrients consisting of 10 ml of fertilizer solution (3 lb. of a 6-12-6 formulation per 100 gal. of water) added semi-weekly.

To test the effect of air and soil temperatures, cuttings were inoculated with isolates 5, 9, or 12 by the root-dip method, and roots of check plants were dipped into water. Cuttings at each of 4 soil temperatures (approximately 15°, 21°, 26°, and 32°C) were placed in greenhouse cubicles maintained at approximately 15°, 21°, 26°, and 32°C air temperature. Experiments with air temperatures at 26° and 32° were run during September and October and those at 15° and 21° during November and December. Each soil-

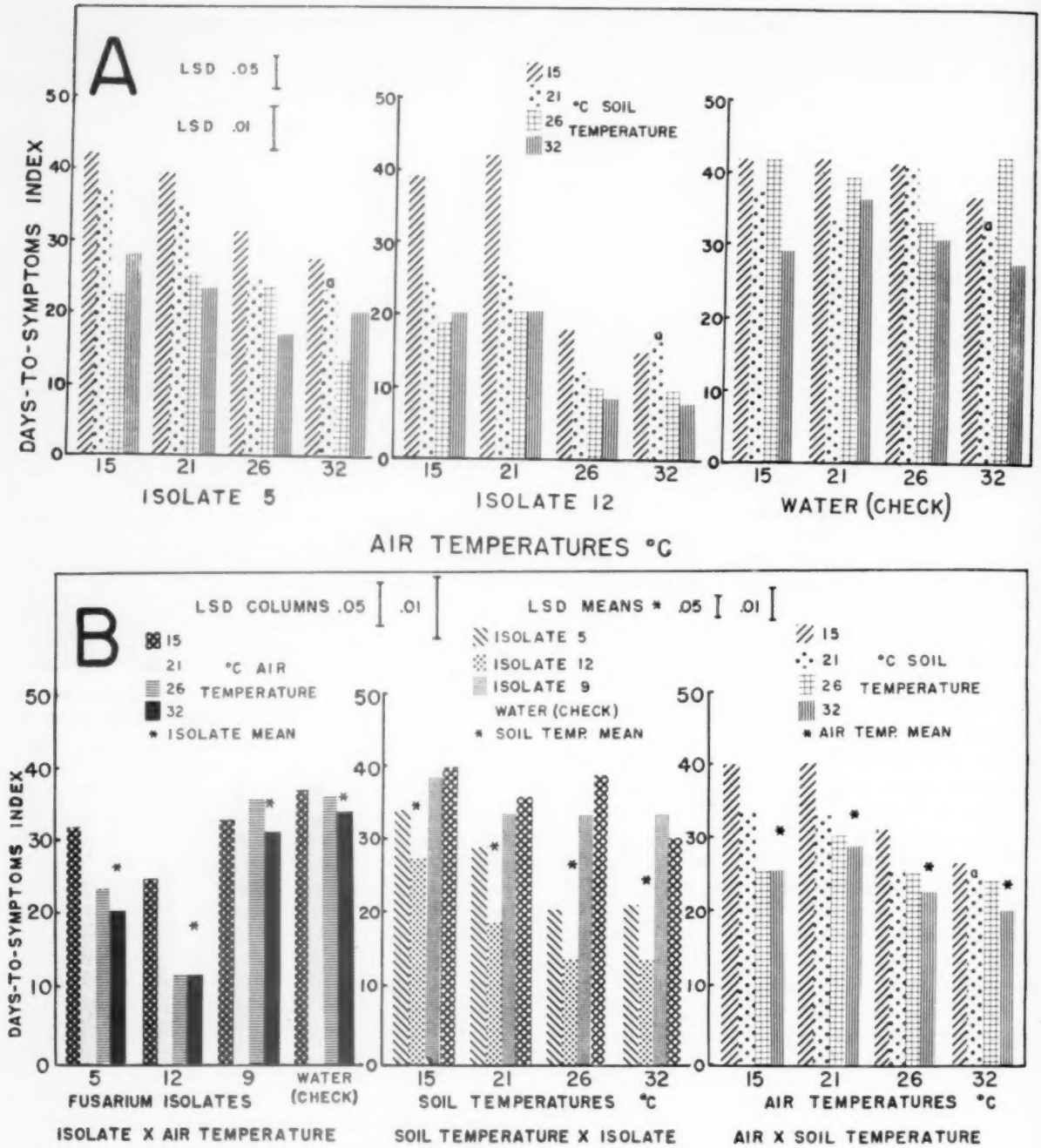

FIG. 2. Days-to-symptoms indexes for Aura carnations inoculated with 3 isolates of *Fusarium oxysporum* f. *dianthi* and grown at various soil and air temperatures for 42 days. Each column represents the mean value for 12 plants. The letter *a* indicates that missing plot values were calculated for this temperature combination. A) Effect of various temperature combinations. B) Interactions of air temperature, soil temperature, and inoculum.

temperature unit was a single Wisconsin-type tank containing 16 randomized glazed crocks, 3 plants per crock and 4 crocks for each of the 4 treatments. Data were recorded for 42 days (Fig. 2). Isolate 9 was nonvirulent in this experiment, hence data for it are not shown in Figure 2, A.

The mean index for isolate 12 was less than that for isolate 5 and both were less than that for the water check; these differences were significant at the 1 per cent level. Plants treated with water showed no significant response to changes in air temperature. With the 2 virulent isolates, 5 and 12, symptoms developed sooner at 26° than at 21° (air temperature). These differences were significant at the 1 per cent

level for isolate 12 and at the 5 per cent level for isolate 5.

Within the range studied, symptoms developed progressively earlier as the soil temperature was increased; however, only the difference between the mean for 15° and that for 26° and the difference between the mean for 15° and that for 32° were significant. With isolate 5, the responses at 15° and at 21° were alike, and each was significantly different from those for 26° and those for 32°C. With isolate 12, there were no differences among the means for 21°, 26°, and 32°, and they all differed significantly from the mean for 15°C. Soil temperature did not affect the host reaction to isolate 9 or to water, except that plants treated with water developed symptoms sooner at 32°C than at other temperatures.

As the air temperature was increased, symptoms developed progressively earlier. There was no significant difference between the mean for 15° and that for 21°, but those for 26° and 32° were significantly lower than that for 21°. At each air temperature tested, each increase in soil temperature usually resulted in a more rapid onset of symptoms. At all air temperatures except 32°, the significant effect of soil temperature was reached at 26°.

Host susceptibility.—In order to obtain some indication as to the susceptibility of greenhouse carnations to different isolates of *F. oxysporum* f. *dianthi*, each of 11 clonal varieties were inoculated with isolates 5, 11, and 14 in a randomized-block design; there were 3 replications, and each treatment unit consisted of 5 plants. The experiment was begun in January and was concluded after 70 days. The varieties My Love, Dairy Maid, Pandora, Barbara Brigham, and Topper were susceptible to each of the 3 isolates. Patten 51 Rose, Beltsville selection 49-38-1, Colonel Blimp, and Tetra Sim were resistant to isolate 5 and susceptible to isolates 11 and 14. The variety Barbara was resistant to isolate 14 and susceptible to isolates 5 and 11. Canary Queen was resistant to isolates 5 and 11 but susceptible to 14. The significant responses of 2 varieties (Table 3) indicate strongly that the 3 isolates represent 3 different pathogenic races of *F. oxysporum* f. *dianthi*.

Seedlings of 10 species and varieties of *Dianthus* were inoculated by the root-dip method with isolate 5 of *F. oxysporum* f. *dianthi* and check plants were dipped in water (Table 4). Isolate 5 gave a high percentage of infection in all the hosts tested. It was of particular interest that *D. barbatus* was highly susceptible to this Fusarium isolate from *D. caryophyllus*. The surviving plants were grown for 1 year and reinoculated when mature plants. The seedling resistance was maintained in the mature plants.

TABLE 3.—*Days-to-symptoms indexes for 2 varieties of carnations inoculated with 3 isolates of Fusarium oxysporum f. dianthi*

Variety	Mean days-to-symptoms index[a]			
	Isolate 5	Isolate 11	Isolate 14	Water
Barbara	33.0	31.2	62.9	64.7
Patten 51 Rose	62.1	34.3	34.5	63.2
LSD (0.05)			9.4	
LSD (0.01)			12.5	

[a] Mean number of days from inoculation to the appearance of symptoms or to the termination of the experiment (70 days). Each figure is the mean for 30 plants.

DISCUSSION.—The main criteria used in identifying isolates of *F. oxysporum* f. *dianthi* were the symptoms produced on inoculated cuttings of carnations. In general the symptoms observed conformed with published descriptions of Fusarium wilt of carnations (2, 7). These same isolates, with high inoculum density and high soil and air temperature, produced a general multilateral discoloration and wilting and early death in a substantial proportion of inoculated plants. The group of isolates that produced typical symptoms were very similar in morphological and cultural characteristics and essentially conformed with descriptions of *F. oxysporum* f. *dianthi* in earlier papers (2, 3, 7). There was variation in pigmentation and in spore size among isolates that produced the same symptoms; therefore, the usefulness of these characteristics in identification of this pathogen is questionable. Snyder and Hansen (6) made similar observations with regard to spore size and Bickerton (2) with regard to pigmentation.

Inoculations by dipping roots of carnation cuttings in inoculum and inoculations in stem wounds proved equally effective and resulted in earlier development of symptoms than did other methods of inoculation tested. This was somewhat in contrast to the report of Bickerton (2), who found stem-wound inoculations to be more effective than was the dipping of roots. The

TABLE 4.—*Percentage of plants showing symptoms in species and varieties of Dianthus inoculated with isolate 5 of Fusarium oxysporum f. dianthi*

Seedling variety or species[a]	No. of plants[b]	Percentage of plants showing wilt symptoms	
		Isolate No. 5	Water
D. caryophyllus			
Perpetual Flowering	40	95	5
Enfant de Nice	40	80	17
Malmaison Giant	40	85	5
D. plumarus			
Type	40	80	0
Spring Beauty	40	70	5
D. chinensis			
var. *heddewigi*	120	72	3
D. barbatus (type)	80	93	13
Dianthus hybrids			
Sweet Wivelsfield	60	80	7
Tetra Heddensis	60	70	7
Alwoodi alpinus	80	65	0

[a] Names are those on the seed packet of the commercial source, except for *D. chinensis* var. *heddewigi*.

[b] Half the seedlings were inoculated with Fusarium, and half were dipped in water.

ease of applying inoculum to large numbers of cuttings by the root-dip method, however, would make it more desirable for large-scale pathogenicity tests. Other inoculation techniques used resulted in delayed symptom development and would be difficult to use. It is of interest that *F. oxysporum* f. *dianthi* became established in sterilized soil and caused a more rapid onset of symptoms that it did when added to unsterilized soil. This may have resulted from lack of competition from other organisms in the sterilized soil. Similar conditions may account for reports from commercial growers of infrequent but severe infection with the Fusarium wilt pathogen after the initiation of a soil-sterilization program. Of the environmental factors tested, only soil and air temperature appeared to be of practical use for reduction of the incubation period in experimental work with this disease.

Since only 1 of 120 noninoculated plants in the test for cross infection developed symptoms during an 80-day period, it was assumed that little or no cross infection occurred when 2-in. pots containing treated plants were placed in the bench such that they touched each other in both directions. Results from this test indicate that either fungus material was not splashed from one pot to another by greenhouse watering or that the fungus material that was splashed was diluted sufficiently so that there was no development of symptoms. These results indicate that similar spacing can be used in tests with *F. oxysporum* f. *dianthi*, thus maximum use can be made of available heated bench space.

Results of the present study confirm the report by Bickerton (2) and observations by commercial growers that hot weather increases severity of Fusarium wilt of carnations. The effect of soil temperature independent of air temperature on Fusarium wilt of carnations is reported for the first time.

The results with the water check plants at soil temperatures between 26° and 32°C (Fig. 2.A) indicated a direct effect of high soil temperatures on newly transplanted carnation cuttings. This finding might cast some doubt on the significance of the extremely short incubation period with isolate 12 at soil temperatures of 32°C (Fig. 2.A); however, similar short periods were obtained with isolate 12 at a soil temperature of 26°, and no effect of this soil temperature on healthy plants was detected. Incubation periods of 9.8 and 9.6 days are the shortest reported for Fusarium wilt of carnations. The best combination of air and soil temperatures for the routine testing of carnations for susceptibility must be economical to establish and easy to maintain and should result in early onset of symptoms. For these reasons, the use of soil temperature of 26°C and of air temperature of either 21° or 26° is indicated.

Existence of pathogenic races of *F. oxysporum* f. *dianthi* was suggested by Bickerton (2) and Guba (4). The present work on host susceptibility within greenhouse varieties of *D. caryophyllus* established the existence of 3 pathogenic races of this fungus. Since the 3 isolates tested represent 3 different races, the possibility of an unlimited number of pathogenic races is suggested. It is obvious that the existence of races and the susceptibility of all species of *Dianthus* tested greatly complicate the problem of breeding carnations for resistance to Fusarium wilt.

F. oxysporum f. *dianthi* was so designated by Snyder and Hansen (6) because of its specificity to the genus *Dianthus*. In 1941, Snyder (5) described *F. oxysporum* f. *barbati* because of its specificity to sweet william (*D. barbatus*). He was unable to obtain infection of any of 12 varieties of carnation (*D. caryophyllus*) with this form. Armstrong and Armstrong (1) extended the host range of *F. oxysporum* f. *dianthi* within the genus *Dianthus* and also found species of 2 other genera of the Caryophyllaceae to be susceptible. They concluded that the species of *Fusarium* causing sweet william wilt "is either the carnation wilt *Fusarium* or a race of this organism with *D. barbatus* as the common host." The present investigation confirmed their report (1) of susceptibility of *D. barbatus*, *D. chinensis* var. *heddewigi* Regel, *D. alwoodi* var. *alpinus* Hort., and *D. plumarus* L. to isolates of *Fusarium* from *D. caryophyllus*. It also extended the host range to include 2 varieties of *Dianthus*, Tetra Heddensis, Sweet Wivelsfield, and 2 garden varieties of *D. caryophyllus*, Enfant de Nice and Malmaison Giant. Both Armstrong and Armstrong (1) and the present workers used seedlings of *Dianthus* in their host-range studies. It is possible that greater susceptibility in the seedling stage accounted for the infection of *D. barbatus* and some of the other species of *Dianthus*. Experiments are underway to test this possibility. The individual plants of the *Dianthus* species that proved resistant to *F. oxysporum* f. *dianthi* as seedlings also proved resistant as mature plants. More tests are being made to determine whether the resistance found in commercial varieties is based on resistance to infection or merely to suppression of symptoms.

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SUPPRESSION BY TWO SUBSTITUTED CARBAMATES OF SYMPTOMS INDUCED BY SOUTHERN BEAN MOSAIC VIRUS[1]

F. M. Porter, B. C. Smale, W. H. Preston, Jr., and J. W. Mitchell[2]

SUMMARY

A study of the effects of antibiotics and other compounds on virus-infected plants demonstrated that certain substituted carbamates suppressed symptom formation in Pinto bean stems freshly inoculated with southern bean mosaic virus. Application of either furfuryl 5-chloro-2-methylcarbanilate or furfuryl carbanilate in lanolin immediately following stem inoculation resulted in almost complete suppression of symptoms without apparent injury to the plants. A "2-phase" carrier system composed of 1 volatile and 1 nonvolatile carrier proved satisfactory for treatments prior to inoculation. Use of furfuryl 5-chloro-2-methylcarbanilate in this carrier system either before or immediately after inoculation resulted in complete suppression of symptoms. This effect was, however, associated with slight localized injury of the stem.

During the past decade, effects of organic compounds on virus multiplication and symptom formation have been reported by many workers (1, 2, 3, 5, 6, 7, 8, 9, 10, 13, 14, 15). Gray (4) obtained marked inhibition of both local lesions and systemic infections caused by southern bean mosaic virus and by tobacco mosaic virus by spraying the inoculated plants with noformicin, but this treatment did not completely prevent symptom development.

The purpose of the present research was to select from a large number of compounds those that would greatly suppress symptom formation in Pinto bean stems infected with southern bean mosaic virus and to learn whether these compounds could be applied to the plant in such a way as to suppress completely symptom development.

METHODS.—The stem-scarification technique used in this research was reported earlier (12). With this technique, chemicals can be tested readily to determine their effect on symptom production. Briefly, the method consists of inoculating localized areas on the stems of Pinto bean plants with the virus. Ninety-five to 100 per cent of the inoculated areas turn brown within 4–5 days, a response associated with multiplication of the virus. Application of an effective chemical in lanolin as a carrier and applied as a thin layer to the freshly inoculated areas reduces the number of areas that become discolored or prevents their discoloration altogether. This reduction in the number of discolored areas was used to indicate the effectiveness of a chemical in suppressing symptom formation.

RESULTS AND DISCUSSION.—*Selection of new symptom-inhibiting compounds.*—Among 529 compounds tested, 1 substituted carbamate, furfuryl 5-chloro-2-methylcarbanilate, was found to suppress symptom development by 98–99 per cent when applied to localized stem infections (Table 1). So far as is known to the authors, this substituted carbamate has not been previously reported as having such symptom-inhibiting properties.

The molecular structure of this compound and the structures of 2 available and closely related compounds are of interest in relation to their symptom-inhibiting properties.

Comparison of the structures of furfuryl 5-chloro-2-methylcarbanilate and furfuryl carbanilate indicates that presence of the chlorine and methyl substituents associated with the phenyl ring structure is not essential for the marked suppression of virus symptom development (Fig. 1, Table 1). Comparison of furfuryl carbanilate with 2-methylfuran indicates, however, that the carbanilate portion of the molecule was required to obtain very marked symptom-inhibiting properties.

The following study was made to learn whether furfuryl 5-chloro-2-methylcarbanilate would react with the virus *in vitro* and thus reduce the ability of the virus to induce symptom development. Four mg of the carbanilate, 0.2 ml of standard virus inoculum, and 4 ml of water were combined to make a mixture that contained 1000 ppm of furfuryl 5-chloro-2-methylcarbanilate. In 1 experiment this mixture was immediately diluted with water (96 ml) to make a concentration of inoculum equal to that used throughout the present study. In a second experiment the mixture was kept at room temperature for 24 hours before it was diluted and used as inoculum. In neither case was symptom development reduced as a result of

TABLE 1.—*Reduction by substituted carbamates of symptom development induced by southern bean mosaic virus in stem tests on Pinto bean*

Compound	Percentage reduction of symptom development in 3 tests				Apparent injury to plants
	1	2	3	Avg.[a]	
Furfuryl 5-chloro-2-methylcarbanilate	98	99	98	98	None
Furfuryl carbanilate	80	100	100	93	None
2-Methylfuran	45	47	31	40	None

[a] Each average is based on 180 inoculated areas, 6 on each of 30 plants.

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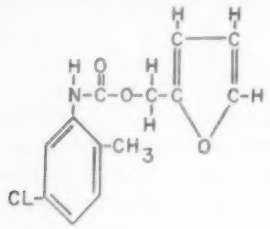
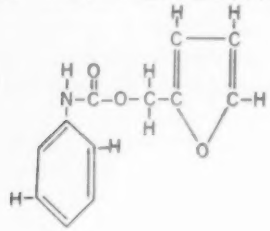
Chemicals were supplied by Spencer Chemical Company, Pittsburgh, Kansas; Columbia-Southern Chemical Corporation, Barberton, Ohio; Stanley A. Hall, U. S. Department of Agriculture, Beltsville, Maryland.

FURFURYL 5-CHLORO-2-METHYLCARBANILATE
(VERY HIGHLY EFFECTIVE)



FURFURYL CARBANILATE
(VERY HIGHLY EFFECTIVE)



2-METHYLFURAN
(MODERATELY EFFECTIVE)

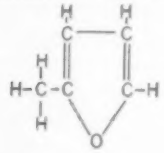


FIG. 1. Structural formulae of 2 substituted carbamates and of 2-methylfuran and the relative effectiveness of the 3 compounds in the suppression of symptoms caused by southern bean mosaic in stems of Pinto beans.

exposing the virus to a concentration of 1000 ppm of the carbanilate.

Although interesting, these data fail to prove that the chemical does not react directly with the virus inside of the plant in such a way as to reduce greatly symptom development. It is, of course, not possible to establish conditions *in vitro* that even approach those that exist inside a cell. For example, it is not at present possible to determine the concentration of the virus nor that of the chemical in a single cell. The concentration of furfuryl 5-chloro-2-methylcarbanilate in these experiments, therefore, had no known relationship to the concentration of the chemical that existed within the cells of the plants.

Effectiveness of furfuryl 5-chloro-2-methylcarbanilate for suppression of symptom development when applied in different carrier systems.—Since the substituted carbamates tested markedly reduced symptom expression, apparently without injuring the plants, but failed to eliminate symptom formation under the conditions used, these compounds were studied in greater detail in attempts to achieve complete suppression of symptoms. Emphasis was placed on the use of furfuryl 5-chloro-2-methylcarbanilate (termed 4067 herein), because it was readily available and highly effective. When the usual screening technique was used, application of 10–15 μg of 4067 in lanolin to each inoculated area reduced symptom expression 98–99 per cent below that of controls. There was no apparent injury to the plants. In these tests, the chemical was applied immediately after inoculation.

Since effectiveness seemed to be associated with absorption of the substituted carbamate by the plants, it seemed possible that treatment of stems with 4067 prior to inoculation might result in absorption of a sufficient amount of chemical to suppress symptom formation completely. The usual method of applying the chemical in lanolin-paste mixtures was not useful for treating the stems prior to inoculation, since it was not possible to inoculate an area covered by the lanolin layer nor was it practical to remove the lanolin just prior to inoculation. Attempts were made, therefore, to develop a carrier suitable for use prior to inoculation.

A "2-phase" carrier system, composed of a volatile solvent in which a small amount of a nonvolatile carrier was dissolved, seemed worthy of trial. In such a system, the volatile solvent dissolves the active compound and thus disperses it molecularly or nearly so. Previous experience with growth-regulating compounds showed that after application to the plant, the volatile portion of such a carrier system evaporates and leaves a very thin layer or minute globules of the nonvolatile portion on the surface of the plant. There also is evidence that in the use of similar carrier systems, regulating compounds (similar in molecular structure to 4067) have been retained by this nonvolatile residue in a finely divided and highly effective state (11).

Since 4067 is not readily soluble in water, both ethanol and acetone were tried separately as major volatile solvents. These solvents do not produce visible toxic effects when applied in relatively small amounts to the surfaces of bean stems. Tween 20 (polyethylene sorbitan monolaurate), Carbowax 400, Carbowax 600 (polyethylene glycols), and Emulphor ELA (polyoxyethylated vegetable oil) were tested as possible nonvolatile carriers. The carbamate was first dissolved in the volatile solvent, then the nonvolatile carriers were added separately in small amounts in the individual tests. All combinations proved to be unsatisfactory, since they permitted precipitation of the substituted carbamate, were toxic to the plants, or seemed to cancel the symptom-inhibiting effect of 4067.

Relation of carriers to symptom development.—Since in the use of the conventional technique, 4067 in lanolin alone markedly reduced virus symptoms when applied to the virus-infected areas, an attempt was made to dissolve such a 4067-lanolin mixture in a volatile solvent and in this way coat the stems with a very thin layer of the effective 4067-lanolin mixture.

Acetone was used as the volatile solvent because lanolin is more soluble in acetone than in ethanol. When the stem-inoculation technique was used, complete suppression of symptom formation was achieved repeatedly with various 4067-lanolin-acetone mixtures by pretreatment and posttreatment of inoculated areas. These mixtures were applied prior to inoculation in 2 tests. Symptoms failed to develop on the treated plants, whereas 98 and 99 per cent of the inoculated areas on untreated plants in these experiments developed the usual symptoms. The substituted carbanilate was applied subsequent to inoculation in 5 other experiments. None of the treated plants developed symptoms, whereas 98–100 per cent of the untreated plants in these tests developed symptoms. In each case, however, use of these mixtures resulted in slight localized injury as evidenced by discoloration of tissues near the inoculated areas. This injury was associated with the use of a lanolin-acetone mixture as carrier. It occurred even when very small amounts of lanolin were used, i.e. 1/128 of the amount of lanolin required to saturate acetone, but no injury was evident when a similar amount of 4067 was applied in either a lanolin carrier or an acetone carrier.

On the basis of these results, it appeared that the amount of 4067 absorbed by the stems from the "2-phase" carrier system was sufficient to induce the injury and to suppress completely development of symptoms caused by the virus. It was assumed that the relatively large intake of 4067 from this carrier system may have been due to the relatively large number of finely divided particles of the substituted carbamate held by the lanolin in contact with the surface of the stem. An attempt was made to determine whether the concentration of finely divided particles of 4067 in the carrier system was in excess of the concentration required for complete suppression of symptom formation. The concentration of 4067 was held constant and the amounts of lanolin and acetone in the mixture were adjusted so that the substituted carbamate would be less finely divided in the carrier system. Lanolin (in which 4067 is not completely soluble) was used as the major carrier, and relatively small known volumes of acetone were added to the 4067-lanolin mixture as a means of adjusting the amount of the substituted carbamate present as particles of relatively small size. With this type of carrier system, the amount of 4067 present as relatively small particles was reduced below that which induced injury, but the mixture failed to suppress virus symptom formation completely.

When the amount of acetone was increased gradually, a point was reached at which very slight injury occurred; however, the mixture failed to suppress symptom formation completely. Further increase in the amount of acetone resulted in still more injury, and again complete suppression of symptom development occurred as in earlier experiments when a comparable amount of injury was evident.

It might be assumed that at least some of the injury connected with the suppression of symptom development obtained with the substituted carbamate dispersed in the "2-phase" carrier were actually due to 4067, to the lanolin, to the acetone, or to a combination of any 2 of these components. As a test of this hypothesis, each component was applied separately and in all possible pairs. In no case was there any apparent injury to the plants. Crystals of 4067 had no apparent effect on symptom development regardless of whether they were placed on the inoculated area before or after inoculation. These results emphasize the fact that the effectiveness of the compound was largely dependent on the type of carrier or carrier system used.

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DAMPING-OFF OF LADINO CLOVER AND LESPEDEZA AS AFFECTED BY SOIL MOISTURE AND TEMPERATURE[1]

J. H. Graham, V. G. Sprague, and R. R. Robinson[2]

SUMMARY

A series of experiments was conducted to determine the pre-emergence and postemergence damping-off of Ladino clover and of common Lespedeza by *Fusarium roseum*, *Rhizoctonia solani*, and *Pythium debaryanum* at constant temperatures from 52°–94°F at 7° intervals and at 3 levels of soil moisture. The temperatures most favorable for radial mycelial growth of Fusarium and Rhizoctonia were near 80° and of Pythium near 87°F. Temperatures for most favorable seedling emergence of Ladino clover were 66° and 73° and for Lespedeza near 87°F.

High temperatures favored the pre-emergence damping-off of Ladino clover by the 3 fungi, and low temperatures favored pre-emergence damping-off of Lespedeza by Fusarium and Pythium. Rhizoctonia reduced the emergence of Lespedeza very little.

Postemergence damping-off of Ladino clover generally was severe in the low temperature range. Rhizoctonia and Pythium damped-off 85–100 per cent of the Lespedeza seedlings at temperatures of 66°–87°F. Fusarium was active against Lespedeza only at the lower temperatures.

In all treatments, Fusarium was most active in moderately dry soil, slightly active in soil with moisture content at field capacity, and practically inactive in saturated soil. In general, damping-off of Ladino clover by Rhizoctonia was highest in soil near field capacity, whereas moisture had little effect on the damping-off of Lespedeza. Pythium generally was most active in wet soil.

Establishment of legumes is a major problem in grassland agriculture. One of the causes for poor emergence and survival of seedlings is damping-off, incited by certain soil fungi. Several factors, such as resistance of the host, pathogenicity of the fungus, soil moisture, and temperature undoubtedly affect the severity of pre-emergence and postemergence damping-off. Gerdemann,[3] in studies at high soil moisture, found that the pre-emergence damping-off of red clover by *Pythium* sp. was most severe at high temperatures. Hanson[4] reported that most pre-emergence and postemergence damping-off of red clover, alfalfa, and sweetclover in naturally infested soil of high moisture occurred at high temperatures. Leach[5] investigated factors affecting pre-emergence damping-off of certain vegetable crops in soil with moisture content at field capacity and found that infection was most severe at temperatures relatively less favorable to the host than to the pathogen as measured by the ratio of their growth rates. Beach[6] in greenhouse experiments in which a disease index based on pre-emergence and postemergence damping-off and root damages was used, agrees in general with Leach, with the qualification "when other environmental conditions are favorable." He also reports that *Pythium ultimum* Trow increased in activity with increased soil moisture up to saturation, whereas *Rhizoctonia solani* Kühn reached maximum activity at $\frac{2}{3}$ saturation.

Since the main factors affecting damping-off have been studied primarily in single-variable experiments, the tests reported here were designed to study the interaction of 3 fungi on Ladino clover (a cool-weather legume) and Lespedeza (a warm-weather legume) at 3 soil moisture levels and at 7 constant temperatures.

MATERIALS AND METHODS.—Potting soil used in the experiments consisted of Hagerstown silt loam to which well-rotted manure, fertilizer, lime, and sand had been added. The moisture contents of this potting mixture at the wilting point, moisture equivalent, and saturation were 9, 27, and 45 per cent, respectively, on a dry-weight basis. Soil was moistened and steamed for 9 hours at approximately 5 lb. pressure the day before each test was started. The soil was then mixed and portions were seeded with the selected fungus by spraying a water suspension of chopped inoculum onto the soil as it was shoveled back and forth. Fungi used were *Pythium debaryanum* Hesse, *Rhizoctonia solani*, and a Fusarium isolate in the group around *F. roseum* (Lk.) Snyd. & Hans. These pathogens were isolated from diseased Ladino clover seedlings in field soil. In the control (no pathogen), a water suspension of some of the common soil saprophytes was sprayed onto the soil.[7] Each lot of soil

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[5] Leach, L. D. 1947. Growth rates of host and pathogen as factors determining the severity of pre-emergence damping-off. Jour. Agr. Res. 75: 161-179.

[6] Beach, W. S. 1949. The effects of excess solutes, temperature and moisture upon damping-off. Pa. Agr. Exp. Sta. Bul. 509, 29 p.

[7] Results from previous tests indicated that saprophytes added to steamed soil reduced recontamination by soil pathogens but did not affect seedling growth.

was placed in glazed crocks 4 in. in diameter and 6–7 in. deep. Fifty seeds of Ladino clover or common Lespedeza were planted in each crock and covered with ¼ in. of loose soil.

The soil moisture was brought to 22–23 per cent before potting. Immediately after seeding, the series run at low soil moisture was covered with transparent plastic. The water content was as low as would give uniformly good germination without soil compaction. For the medium moisture level, water was added to bring the soil to the moisture equivalent. Saturation was obtained by adding enough water to produce slight drainage from the bottom of the crock. After water was added, all crocks were covered with clear plastic to prevent water loss by evaporation. The plastic covers were removed after emergence of the seedlings, and stand counts were taken to determine pre-emergence damping-off.

The same crocks were used to determine the amount of damping-off among the emerged seedlings. Differences in stand did not appear to be an important factor in postemergence damping-off. It was impossible to maintain uniform low soil moisture after the crocks were uncovered. The series at lowest moisture was allowed to become dry on the surface and remain dry for about 1 day, then water was added to moisten the soil to a depth of 2–3 in. The crocks at the medium soil moisture level were watered shortly before the soil surface normally would have become dry. Saturated soil was maintained by watering daily.

Duplicate crocks of each soil moisture, host, and pathogen were placed into each chamber at controlled air temperatures from 52°–94°F at 7° intervals. Since but 4 chambers were available, only 4 different constant temperatures could be maintained at 1 time, and it was necessary to make 2 runs to complete a series. Four runs (2 complete series) provided 4 replications of each treatment.

Relative humidities were adjusted to obtain similar vapor pressure deficits (VPD), i.e. equal rates of water loss by evaporation in each chamber. The VPD was approximately 0.25 in. of mercury.

"Daylight" and "white" fluorescent lamps provided light of approximately 500 foot-candles for 12 hours each day.

To obtain relative figures indicating the growth ratio of each legume at the 7 temperatures, the coefficient of velocity of emergence (C.V.E.) was calculated by the Kotowski[8] formula:

$$\frac{\text{Total emergence at end of period}}{\text{Sum of (each daily emergence increase} \times \text{days since planting)}} \times 100 = \text{C.V.E.}$$

Since this formula does not compensate for the difference in total emergence, the numbers of seedlings emerged in 4 replications and 3 moisture levels were averaged and are presented for comparison (Table 1). The data used to calculate the C.V.E. and total emergence were obtained from the control series. In other tests, emergence of seedlings in soil in Petri dishes agreed closely with emergence in crocks of soil.

TABLE 1.—*Percentage germination and coefficient of velocity of emergence (C.V.E.)[a] of Ladino clover and Lespedeza at various temperatures[b]*

Temperature (°F)	Ladino clover		Lespedeza	
	Percentage germination	C.V.E.	Percentage germination	C.V.E.
52	64	12	89	8
59	67	18	89	12
66	65	22	89	16
73	69	21	88	20
80	70	19	91	25
87	44	19	89	30
94	13	14	78	27

[a] Calculated by the Kotowski formula (see text).
[b] Each figure represents average of 4 replications and 3 moisture levels.

For determining the rate of growth of the 3 fungi, average radial growth per 24-hour period on agar was used. The radius of growth from a locus seemed more important in causing damping-off of nearby seedlings than the density of mycelial growth, which would be obtained by weighing mycelial mats.

The data were analyzed as a split-plot design with temperatures as the main plots and pathogens, hosts, and moisture levels as subplots. The data obtained at 94°F were not included in the analysis because emergence of Ladino clover was greatly reduced even in the absence of pathogens.

RESULTS.—Total emergence of Ladino clover was relatively uniform (64–70 per cent) between 52° and 80°F and decreased sharply at higher temperatures. For Lespedeza, emergence ranged from 88–91 per cent between 52° and 87°F (Table 1). Total emergence of either legume was about the same at the 3 moisture levels, except that germination of Ladino clover was reduced approximately 50 per cent at the 2 highest temperatures.

The calculated rate of emergence (C.V.E.) of Ladino clover was highest at 66°F and decreased slightly near the extremes, whereas for Lespedeza the C.V.E. increased up to 87° and then dropped only slightly.

The optimum temperature for radial growth of mycelium on agar was 87°F for Pythium and 80° for Rhizoctonia and Fusarium (Table 2). Good growth of the 3 fungi occurred from 66°–87°.

Statistical analysis of the data both on pre-emergence and postemergence damping-off of the 2 legumes gave highly significant *F* values for all treatments and interactions except temperature and the pathogen × host interaction in pre-emergence. The highly significant interactions of temperature with host,

[8] Kotowski, F. 1927. Temperature relations to germination of vegetable seed. Amer. Soc. Hort. Sci. Proc. 23: 176-184.

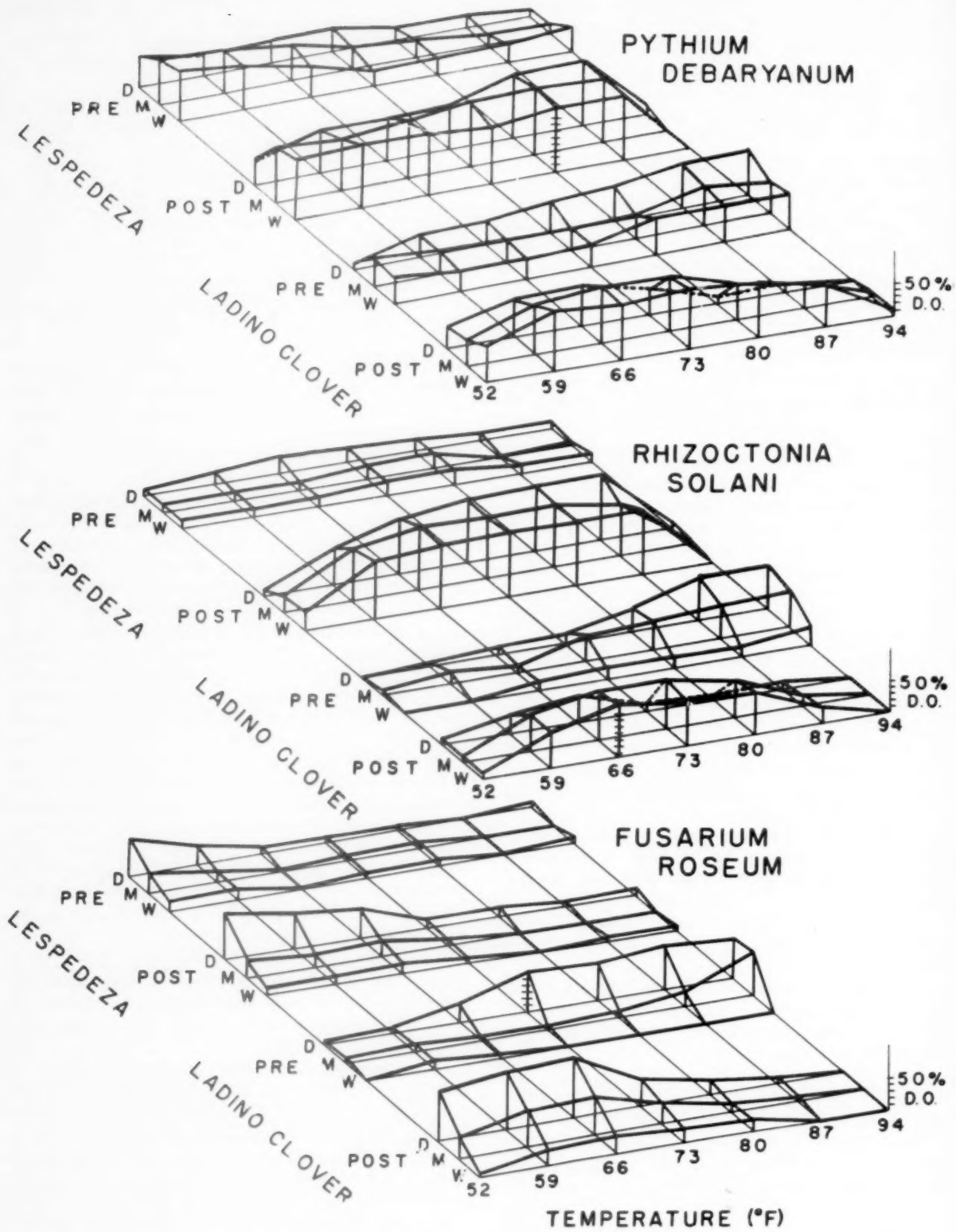

FIG. 1. Percentage damping-off of Lespedeza and Ladino clover by 3 fungi at various temperatures and soil-moisture levels.

TABLE 2.—*Average mm of mycelial growth per 24 hours of 3 damping-off fungi at 7 temperatures*

Temperature (°F)	*Pythium debaryanum*	*Rhizoctonia solani*	*Fusarium roseum*
52	1	2	2
59	6	6	3
66	20	20	5
73	29	21	7
80	32	22	8
87	39	21	4
94	17	4	1

pathogen, and moisture account for the lack of significance of temperature alone. This also applies to the first order interaction of the pathogen × host.

Pythium caused pre-emergence damping-off of both legumes at all temperatures; on Lespedeza, injury was greatest at low temperatures, whereas on Ladino clover it was greatest at high temperatures (Fig. 1). The effects of soil moisture were small.

Postemergence damping-off of Lespedeza by Pythium was high, particularly in the wet soil and at moderately high temperatures. Even in dry soil, damping-off was severe at 80° and 87°F but almost absent at 94°F.

Postemergence damping-off of Ladino clover by Pythium was most severe at intermediate temperatures (66° and 73°F) and was lowest at high temperatures (87° and 94°F). High soil water increased damping-off.

Rhizoctonia caused no appreciable pre-emergence damping-off of Lespedeza at any temperature and only slight injury to Ladino clover except under conditions of high temperature and low soil moisture. Increased postemergence damping-off of both legumes occurred at all temperatures except the highest and lowest. Medium soil moisture favored postemergence damping-off of Ladino clover; with Lespedeza, soil water had little effect.

Both pre-emergence and postemergence damping-off of Lespedeza by Fusarium was negligible except at low temperature and even then was slight except in dry soil.

Damping-off of Ladino clover by Fusarium presented an interesting contrast in occurrence of pre-emergence damping-off only at high temperatures and postemergence damping-off only at low temperatures. In both cases, damping-off was confined primarily to the plants in dry soil.

Since the amount of pre-emergence damping-off of the hosts by the 3 fungi varied with the treatments, the initial stands for determining the percentage of postemergence damping-off were different. It might be expected that if more seedlings were in the crocks because of less pre-emergence damping-off, conditions would be more conducive for postemergence damping-off. Inspection of the data indicates that this was of minor importance. Also, since only 50 seeds were planted and fewer emerged, the seedlings were not crowded in any case.

Fusarium caused a different type of seedling disease than did Rhizoctonia or Pythium. Fusarium attacked the roots instead of the hypocotyls at the soil level; the latter is typical for postemergence damping-off.

Almost no postemergence damping-off by any fungus occurred at 94°F. Germination of Ladino clover was greatly reduced, and the prolonged periods of high temperature caused many of the seedlings of Ladino clover and Lespedeza to shrivel.

DISCUSSION.—The results of these trials indicate that many interacting factors determine the severity of pre-emergence and postemergence damping-off of Ladino clover and Lespedeza. Pre-emergence damping-off of both legumes was more closely correlated with the effects of temperature on the host than on the fungi. In general, emergence was lowest at the temperatures least favorable for the host. In contrast, postemergence damping-off tended to be highest at the temperatures most favorable for the hosts. Two marked exceptions occurred with Fusarium, which caused both pre-emergence and postemergence damping-off of Lespedeza at low temperatures and no postemergence damping-off at 94°F even though the growth of the fungus was not inhibited at this temperature. Because of radiation from the lights, temperatures at the soil surface may have been slightly higher than the 94° air temperature. Spot tests in other recent experiments in the chambers have indicated such differences between soil and air temperatures.

Soil moisture markedly affected the severity of damping-off. It is not surprising that Fusarium was active only in moderately dry soil, since the fungus commonly causes a dry rot of many crops.

These data indicate the importance of knowing the principal pathogenic fungi in greenhouse soil, since a certain level of soil moisture may control one pathogen and favor another. It is postulated that greenhouse temperatures also might be regulated to reduce seedling diseases. If the temperature were kept below 66°F until Ladino clover seedlings had emerged and then raised to approximately 85°F for a short period until danger of damping-off was passed, seedling loss might be reduced appreciably. These temperatures would need to be reversed for Lespedeza.

Neither pre-emergence nor postemergence damping-off could be correlated consistently with the ratio of velocity of emergence and growth rate of the pathogens as used by Leach.

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A PHYTOPATHOLOGICAL NOTE

The Culture of Phytophthora infestans.[1] H. DAVID THURSTON.[2] *Phytophthora infestans* (Mont.) d By grows poorly on many culture media and loses virulence quickly upon repeated transfer. After a number of media and methods had been tested, the following techniques for growing and maintaining it were adopted at the University of Minnesota.

The fungus grew best on lima bean agar of the following formula: 15 g of very finely ground dry lima beans, 10 g dextrose, 10 g agar, 2 g Difco Yeast Extract, and water to make 1 liter. The lima bean powder was suspended in 800 ml of water and autoclaved 45 minutes at 121°C in a container large enough to avoid boiling over. The remaining materials were then added and the mixture heated over an open burner until the agar was melted. The mixture was not strained. Tubed or bottled lima bean agar was then autoclaved at 121°C for 30 minutes. No more than 250 ml of the agar should be put in a liter container or it usually will boil over.

To delay loss of virulence, cultures were transferred only once a month. When the cultures were growing vigorously (5–6 days after transfer), they were stored at 3–5°C. Isolates used very infrequently were stored under mineral oil according to the method of Wernham.[3] The following record of viability illustrates the success of this method.

On February 25, 1951, 5 isolates (races 1; 2; 4; 1.2; and 1.4) were placed under mineral oil; on February 26, 1953, they were transferred to lima bean agar where they grew normally. Sporangia from these isolates were able to infect potatoes with no apparent loss of virulence and no change in their racial identity. Forty stock isolates have been stored under mineral oil since 1953 and none has been lost.

It is difficult and time consuming to obtain large amounts of inoculum of *P. infestans* from lima bean agar or from plants in the greenhouse. Of a large number of materials tested for this purpose, the best found during this study was sterilized yellow peas, as suggested to the author by Dr. Reiner Bonde. Dried yellow peas were first soaked overnight in water, and a layer of the soaked peas 1 in. deep was placed in 250-ml Erlenmeyer flasks. Enough water was added to barely cover the peas, and the flasks were autoclaved at 121°C for 40 minutes. The fungus can be transferred to the flasks either as pieces of mycelium directly from lima bean agar or by pipetting a suspension of sporangia onto the peas. The latter method resulted in the most rapid growth, but even with the first method, the fungus covered the surface of the flask after a week to 10 days at 20°C. Water was added to the flasks to make the sporangial suspension for inoculation. Inoculum from 6 flasks suspended in 2 gal. of water was sufficient for heavy inoculation of all potato plants in 2400 ft. of row. The maximum number of infective sporangia were obtained from yellow peas 10 days to 2 weeks after inoculation. Sporangia from peas 1 month after inoculation still caused a little infection, but after 1½ months, none were able to infect—Department of Plant Pathology and Botany, Institute of Agriculture, University of Minnesota, St. Paul 1, Minnesota.

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[3] Wernham, C. C. 1946. Mineral oil as a fungus preservative. Mycologia 38: 691-692.